



PHARMACOPOEIA

OF THE PEOPLE'S REPUBLIC OF CHINA

(2005)

Volume III

Chinese Pharmacopoeia Commission

People's Medical Publishing House



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Contents

Membership of the 8th Pharmacopoeia Commission of the People's Republic of China	III
Editorial Board of Pharmacopoeia of the People's Republic of China (2005) Volume III	VI
Preface	VII
History of the Pharmacopoeia of the People's Republic of China	XI
New Monographs Included in Volume III (2005)	XIX
Monographs Deleted from the Previous Editions	XX
Appendices Added, Revised and Deleted in Volume III (2005)	XXV
Revised Titles in Volume III (2005)	XXIX
General Notices	XXXII
General Requirements	1
Monographs	25
Appendices	A-1
Index	I-1

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Preface

This edition of the Pharmacopoeia of the People's Republic of China (known as *the Chinese Pharmacopoeia 2005* or in abbreviation as Ch. P 2005) has been prepared in accordance with the principles and designed plan decided by the Eighth Chinese Pharmacopoeia Commission and accomplished with the effort made by Commission members and its Secretariat over more than two years. *The Chinese Pharmacopoeia 2005* adopted by the Executive Commission of the Chinese Pharmacopoeia Commission is approved for implementation by the State Food and Drug Administration of China. This is the eighth edition of Chinese Pharmacopoeia since the founding of the People's Republic of China.

The Chinese Pharmacopoeia 2005 has been considerably revised and improved in General Notices, Requirements of Monographs, General Requirements for Preparations in Appendices and new testing methods, etc. Based on the introduction of advanced technology and experimental methods widely adopted in China and abroad, the contents of Appendices are revised by and large in consistence with those nowadays applied internationally for drug quality control. On the premise that every effort should be made to follow the principle of "safety for use, reliability of therapeutic effect, feasibility of processes, controllability of manufacturing quality and perfection of specification", the monographs admitted in the Pharmacopoeia on the whole reflect the actual clinical use of drugs in China. Furthermore, stylistic rules and layout, wording, units, and symbols, etc. have also been standardized.

The Chinese Pharmacopoeia 2005 is published in three volumes. Volume I contains monographs of Chinese materia medica and prepared slice, vegetable oil/fat and its extract, Chinese traditional patent medicines, single ingredient of Chinese crude drug preparations etc.; Volume II deals with monographs of chemical drugs, antibiotics, biochemical preparations, radiopharmaceuticals and excipients for pharmaceutical use; Volume III contains biological products. *The Requirements for Biologics of the People's Republic of China* is now incorporated into the Chinese Pharmacopoeia for the first time. Based on the characteristics and the needs of improvement of Chinese traditional medicines, chemical drugs and biological products, the research work and the studies are carried out in an active way on drug standards and methodology. At the same time, great efforts are made to unify the national drug standards and to bring them in line with the international standards progressively. In addition, emphasis have been put to harmonization in Appendices of different volumes of Pharmacopoeia, to a sound connection between individual monographs and the relevant appendices, and to the standardization of text wording so as to make this edition more precise and better structured.

The increase of the number of monographs in *the Chinese Pharmacopoeia 2005* is obvious which contains up to 3214 monographs of drugs, with 525 new admissions in total. Volume I contains 1146 monographs, with 154 new admissions and 453 revised; Volume II deals with 1967 monographs, with 327 new admissions and 522 revised; Volume III contains 101 monographs, with 44 new admissions and 57 revised. 9 monographs adopted in *the Chinese Pharmacopoeia 2000* are not admitted in this edition and

123 monographs adopted in *the Requirements for Biologics of the People's Republic of China 2000* and in its *Supplement 2000* are not admitted in this edition.

The number of monographs in Appendices of this edition is much expanded. There are 98 monographs admitted in Appendices of Volume I with 12 new admissions, 31 revised and 1 deleted. There are 137 monographs in Appendices of Volume II with 13 new admissions, 65 revised and 1 deleted. There are 140 monographs in Appendices of Volume III with 62 new admissions, 78 revised and 1 deleted. The monographs in Appendices common to all the three volumes are presented in each volume respectively in a harmonized and unified form.

Extensive applications of contemporary techniques of analysis are shown in this edition. In Volume I, the number of monographs adopting thin layer chromatography in the test for identification reaches 1523 and 45 monographs for content determination; the number of monographs adopting high performance liquid chromatography (HPLC) reaches 479 and 518 items are involved; and the number of monographs adopting gas chromatography in the tests for identification and content reaches 47. In Volume II, the number of monographs, including testing items, adopting HPLC reaches 848 with an increase of 566 in comparison with that in 2000 edition, and this method is mostly used for the analysis of complex formulation and those drugs containing much more interfering factors such as impurities or excipients, and used for content determination in 223 newly admitted monographs. The number of monographs required for identification by infrared analysis reaches 70; the tests for dissolution and test of content uniformity are added in test items in 93 and 37 monographs, respectively, and the test for related substances is added in 226 monographs, and the requirements for systematic suitability testing are more reasonable. Based on the validation of methodology, the test for bacterial endotoxins instead of pyrogen test in rabbits is introduced to 73 monographs; on the premise that the drug purity is ensured, the test for undue toxicity for 42 monographs is deleted.

Significant revisions and expansion are made in the Appendices of this edition leading a great improvement in the monographs of Appendices. In order to adapt the need of drug administration, new preparations such as implants, rinsing agents, enemas, paint, and smeared films, etc. are admitted in the General Requirement for Preparations. Many subtypes of dosage form are also admitted in General Requirement for Preparations, such as soluble tablets and vaginal effervescent tablets admitted into General Requirement for tablets; sustained release capsules and controlled release capsules admitted into General Requirement for capsules, etc. Test for sterility is added in test items in some of preparations of General Requirement for Preparations. New general testing methods have been admitted, such as Determination of Total Organic Carbon in the Water for Pharmaceutical Use, Test for Visible Particles in Injections, Mass Spectrometry, Determination of Cataplasms Adhesion, Test for Allergen, Biological Assay of Calcitonin and Growth Hormone, etc. Furthermore, considerable revisions are made for a number of Appendices according to modern techniques and practical situation, for example, the tests on 12 pesticides containing organic phosphorous and 3 pesticides containing pyrethroid are added for determination of pesticide residues; the test for small volume of injection is included in the Test for Particulate Matter in Injections; the test for system suitability is admitted for Thin-layer Chromatography; Microbial Limit Tests is revised according to the requirements of drug administration route and tests for validation are added; the time of incubation in sterility test has been changed from 7 to 14 days.

In the section of guidelines, such guidelines have been revised to keep pace with the development of

research and production of drugs as the Guidelines the Stability Testing of Drug Substances and Preparations and the Guidelines for Sustained, Controlled and Delayed Release Preparations, and new guidelines are admitted such as the Guidelines for Hygroscopicity and Guidelines for Near-infrared (NIR) Spectrophotometry, etc. Although those guidelines do not serve as legal requirements, they play an important role in assessment of drug quality, in establishing, standardizing and implementing uniform pharmaceutical specification of drugs and medicines.

The safety of pharmaceuticals is another important issue of *the Chinese Pharmacopoeia 2005*. In Appendices of Volume I, for example, atomic absorption spectrophotometry or inductively coupled plasma mass spectrometry is introduced to determine 6 kinds of heavy metals and deleterious elements, and the limits for lead, cadmium, mercury, arsenic and copper are stipulated for the first time; the harmful solvents, such as benzene etc. used in pharmaceuticals should be substituted by other solvents as far as possible. The Guidelines for Application of Safety Tests for Injection of Traditional Chinese Medicine is also admitted in this edition; In Volume II, the Test for Particulate Matter in Injections is applied to 126 Injections intended for intravenous injection; the number of monographs adopting the Test for Bacterial Endotoxin reaches 112; the Determination of Residual Solvent includes the requirements of the International Conference on Harmonization (ICH) for residual solvents, and the test is required for 24 drug substances; the Guidelines for the Analysis of Impurities in Drugs, Guidance for the Quality Control of Positron Emission Tomographic and Technetium [^{99m}Tc] Radiopharmaceutical Preparations are also admitted. In Volume III, new methods such as Determination of Reverse Transcriptase Activity and Test for Residual Aluminum Content in Human Albumin etc. are admitted, and some test methods are improved such as the test for residual bovine serum albumin and test for residual CHO cell protein, etc. On consideration of the status quo of medical industry and practical situation of drugs for clinical use, the requirements set forth in Detailed Regulations for Clarity Test and Criteria formerly issued by the Ministry of Health are replaced by the method for Determination of Visible Particles in this edition so as to enhance the safety of pharmaceuticals including injections.

According to the theory of traditional Chinese medicine—diagnosis and treatment based on an overall analysis of the illness and the patient's condition, the *Indications* under the Chinese patent preparations have been scientifically standardized, so as to avoid the phenomenon of easy misleading usage of drugs and to give prominence to features of the above theory. At the same time, the attention should be paid to the fact that a phenomenon of “different diseases having same syndrome” and “same disease having different syndromes” exists between “Syndrome” in traditional Chinese medicine and “Disease” in Western medicine. The close combination of “Syndrome” in traditional Chinese medicine and corresponding “Disease” in Western medicine embodies the scientificity and accuracy of the expression of *Indications*, so as to ensure clinicians to understand the *Indications* precisely and make use of drugs rationally, thus facilitating the sound development of traditional Chinese medicine in the new era.

The working procedures for preparation of this edition also has been improved. In addition to the traditional way of requesting for comments, the contents of revised appendices and monographs should be publicized on the website of the Chinese Pharmacopoeia Commission for three months, aiming at collecting comments widely from various institutions and organizations. All the feedbacks and inputs should be reviewed by the relevant subcommittee to ensure the feasibility and practicability of the standards and methods revised in this edition of Pharmacopoeia, and ensure that the principle of “openness, justice and fairness” is kept in the process of compiling and editing.

In order to make it easy for reading, *the Chinese Pharmacopoeia 2005* adopts the half-measure in its layout for the first time, and the improvement of quality in printing and binding makes this edition look more elegant.

Thanks to the well-organized work by the Secretariat of the Chinese Pharmacopoeia Commission, the joint efforts made by all the participants and the support from institutions and organizations concerned, the compilation of *the Chinese Pharmacopoeia 2005* proceeded smoothly and the goal is reached as designed despite the heavy work schedule and high requirements for the task. Now *the Chinese Pharmacopoeia 2005* is presenting its new style in front of the world and will play a greater role both in initiating new prospect of national drug administrative work and in the development of the medical industry in China.

郑筱萸

Chairman, the eighth Chinese Pharmacopoeia
Commission of the People's Republic of China

(December 2004)

History of the Pharmacopoeia of the People's Republic of China

The Chinese Communist Party and the Chinese Government have attached great importance to medical and health care of the Chinese people. The People's Republic of China was founded on October 1, 1949 and right in November of that year the Ministry of Health convened a meeting of medical and pharmaceutical experts in Beijing on the compilation of a pharmacopoeia. In January 1950, the Ministry of Health invited Professor Meng Mudi, a well-known pharmacist from Shanghai, to take up the responsibility for the establishment of the Editorial Commission of Pharmacopoeia of China and its secretariat to deal with daily work concerning the compilation of such a compendium for new China.

In April 1950, a working seminar was held in Shanghai, at which the principles and guidelines on the selection of monographs were discussed and the monographs to be included in the Pharmacopoeia were decided. It was recommended under the direction of the Ministry of Health that the new Pharmacopoeia should be compiled in such a way that it is in conformity with the Chinese situations and that it should be nationalistic, scientific and popular in nature. Thereafter, The Ministry of Health invited 49 experts as members and 35 as correspondent members of the Commission who were appointed to 8 panels (nomenclature, chemicals, pharmaceutical preparations, medicaments of plant origins, biological products, medicaments of animal origins, pharmacology, and dosage) respectively. Health Minister Li Dequan served as the chairperson of the Commission. The first Editorial Commission of Pharmacopoeia of the People's Republic of China was thus formally established.

The first Editorial Commission meeting composed of all members was held in Beijing April 24-28, 1951, where resolutions were made on the title of the Pharmacopoeia, list of selected monographs, the nomenclatures, units of measurement and weights, format, the order of arrangement etc. Based on recommendations from the Commission meeting, the draft of the pharmacopoeia was then revised by the Secretariat and submitted to the Ministry of Health for review and the Culture and Education Commission of the State Council for approval at the end of 1952. The Ministry of Health published the first *Chinese Pharmacopoeia* in 1953.

The Chinese Pharmacopoeia 1953 edition contained 531 monographs of substances and articles, including 215 chemicals, 65 medicaments from plant origins, oils and fats, 13 medicaments from animal origins, 2 antibiotics, 25 biological products, and 211 pharmaceutical preparations. After the publication of the Pharmacopoeia the first addendum of the 1953 edition was published in 1957.

The Ministry of Health set up the second Chinese Pharmacopoeia Commission in 1955, with 49 members and 68 correspondent members. Due to various reasons, this Commission failed to fulfill its mission. The third Commission was established in 1957, with 80 members (no correspondent members appointed) and

Professor Tang Tengan, a well-known pharmaceutical chemist, as its chairman. The first meeting of the third Commission was convened from July 28 to August 5 of the same year. Health Minister Li Dequan pointed out at the meeting that it was a big flaw that the first Pharmacopoeia did not cover Chinese traditional medicines that the Chinese people were so used to. At the meeting principles in compiling a Pharmacopoeia were made and nature and purpose of such a compendium were discussed and constitution of the Commission was revised. It was agreed unanimously to admit well-defined Chinese traditional medicines to the Pharmacopoeia. On August 27, six expert committees and a panel under the Commission were approved and set up by the Ministry of Health, namely, the committees of medicines, chemicals, pharmaceutical preparations, biochemicals, pharmacognosy and biological products, and a panel of nomenclature. Under the Commission a Standing Committee was organized, however, routine work in general was dealt with by its Secretariat.

In 1958, it was recommended by the Standing Committee and approved by the Ministry of Health to invite 8 doctors of Chinese traditional medicine and 3 experts of Chinese traditional medicaments as members of an expert committee dealing with the quality specifications of crude drugs used as Chinese traditional medicaments and Chinese patent preparations. Collaborative efforts were made by experts in this field in many parts of this country to incorporate the theory and practical experience of Chinese traditional medicine into the monographs concerned.

The second meeting of this Commission was held in Beijing from June 25 to July 5, 1959. A list of monographs being admitted to the new Pharmacopoeia was proposed and the draft texts reviewed in detail by the expert committees concerned. The work was accomplished in 1962. The State Council approved the publication of the Pharmacopoeia of the People's Republic of China 1963 edition. On January 26, 1965, the Ministry of Health issued a document for *the Chinese Pharmacopoeia 1963* edition and the relevant provisions for its implementation.

The Chinese Pharmacopoeia 1963 edition contained 1310 monographs in its two volumes, each with separated General Notices and relevant appendices. 446 monographs of commonly used Chinese traditional medicaments and 197 monographs of Chinese traditional patent preparations were admitted to Volume I, and 667 monographs of chemical drugs were admitted to Volume II. Additionally, "therapeutic function and chief indication" were stated in the monographs admitted to Volume I and "action and use" of those admitted to Volume II.

The Chinese Pharmacopoeia Commission stopped functioning in 1966 due to the turmoil caused by the "Cultural Revolution". On April 28, 1972, the State Council agreed to the suggestion in the report of the Ministry of Health that "the Commission should be re-established with the participation of Ministries of Health, Petroleum and Chemical industry, Commerce and the PLA's Ministry of Health, headed by the Ministry of Health". A working meeting of the Chinese Pharmacopoeia Commission was convened under the above direction from May 31 to June 10 of the same year in Beijing. 88 representatives of various competent authorities and organizations, including all Provincial (Autonomous regional or Municipal) Departments of Drug Policy and Management, Institutes for Drug Control and others attended the meeting. The focus of the meeting was on the guiding principle, working process, requirements and objects of the editing of the national Pharmacopoeia. The revision plan was recommended after exchange of past experiences in various aspects. Arrangements were made for the drafting of individual monographs by the organizations concerned. The second meeting of the Chinese Pharmacopoeia Commission was held

in Beijing in April 1973. Some guidelines, basic requirements of the Pharmacopoeia and sample monographs for Chinese traditional medicaments and modern medicinal substance as well as the respective explanatory notes had been well discussed and appropriate recommendations were made. The drafting of individual monographs was rearranged according to the place of origin of Chinese medicines and the conditions of pharmaceutical production. On October 4, 1979 the Ministry of Health promulgated that *the Chinese Pharmacopoeia 1977* edition would come into use on January 1, 1980. The total of monographs contained in the 1977 edition is 1925. In Volume I, 1152 monographs were admitted, including 882 monographs of Chinese herbal drugs in general used and in the region of national minorities, extracts of Chinese herbal medicines, oils and fats and some preparation made of single medicinal ingredient. 270 monographs of Chinese traditional patent preparations (including those preparations used in the region of national minorities) were also admitted in the Volume I. 773 monographs of chemicals and biological products etc. were admitted in Volume II.

In 1979 the Ministry of Health invited 112 experts as members to form the fourth Chinese Pharmacopoeia Commission, and Health Minister Qian Xinzong was its Chairman. The first plenary meeting of that Commission was held from Nov. 22 to Nov. 28 in the same year in Beijing. Discussion and amendment were made on the constitution of the commission, provisions for the management of specifications for pharmaceutical preparations and its working plan in the meeting. Ten specialized advisory groups were appointed in the fields of Chinese traditional medicine, Chinese traditional medicaments, medicine and pharmacology, chemicals, biochemicals, pharmaceutical preparations, antibiotics, biological products, radiopharmaceuticals and nomenclature respectively. Monographs being admitted to the new Pharmacopoeia were recommended by the advisory groups concerned. The advisory group on Chinese traditional medicine had the responsibility to review and select the range of monographs to be included in Volume I and the advisory group on medicine and pharmacology had the same responsibility for Volume II. The institutes for drug control and competent authorities or organizations in the locality (provinces, autonomous regions and municipalities), where the drug substance concerned was produced provided draft text of individual monograph with prominent experience and excellent quality. Coordinated review and technical validation were organized by the Secretariat. Some monographs were drafted only after the completion of objective collaborative studies as required. Finally, members of respective advisory groups and representatives of institutes for drug control and drug manufacturers concerned reviewed the draft text, and then sent to the Ministry of Health for approval. *The Chinese Pharmacopoeia 1985* edition was published in September 1985 as planned. The effective date was set on April 1, 1986 as approved by the Ministry of Health. In this edition of Pharmacopoeia, 1489 monographs of drugs were admitted. 506 monographs of Chinese traditional medicaments, crude drugs, vegetable oils and fats and preparations of single ingredient, 207 monographs of Chinese traditional patent preparations, totally amount to 713 monographs were admitted to Volume I. 776 monographs of chemicals, biological products etc. were admitted to Volume II.

The "Drug Administration Law of the People's Republic of China" came into effect on July 1, 1985. It stipulated that "the quality of drugs and medicines must comply with a national, provincial, autonomous regional or municipal standard", and that "the Pharmacopoeia of People's Republic of China and standards for drugs and medicines published and promulgated by Ministry of Health of the State Council are national standards for drugs and medicines". It further stated, "The Chinese Pharmacopoeia Commission subordinate to the Ministry of Health of the State Council is responsible for the stipulation and revision of national standards for drugs and medicines". It defines clearly the official nature of standards for drugs

and medicines and responsibilities of the Commission.

In 1986 the Ministry of Health reorganized the Chinese Pharmacopoeia Commission in accordance with its constitution and invited 150 experts as members of the fifth Commission with Health Minister Cui Yueli as Chairman. The office dealing with routine work was changed to a system with the Secretary General as its chief executive officer. The first meeting of this Commission was convened May 5-8 of the same year. The constitution of the Commission was revised. Comments were made on the task of drug standardization during the seventh Five-year Plan for National Reconstruction. The guidelines and principles of the 1990 edition of the pharmacopoeia were discussed and agreed at the meeting. Panel meetings on Chinese traditional medicaments, Chinese patent preparations, chemicals, antibiotics, biochemicals and pharmacology were held respectively for the tasks of drafting different parts of the next edition and necessary research projects to be carried out. The addendum to *the Chinese Pharmacopoeia 1985* edition was published in November 1987 with 23 new admissions and 172 specific monographs and 21 general monographs were revised or amended. Based on *the Chinese Pharmacopoeia 1985* edition, its first English version was formally published in October 1988. Also published in that year were the selected notes in Volume II. By March 1989, the draft text of the new pharmacopoeia was basically ready for comments through the efforts made by various organizations and associations. The Secretariat of the Chinese Pharmacopoeia Commission was authorized to organize reviewing and editing. In December 1989, an extended meeting attended by the chairman, vice-chairmen of the Commission and the chairmen of all advisory groups was held in Beijing. It was recommended to send the draft text to the Ministry of Health for comments and approval. The Ministry of Health then publishes it as *the Chinese Pharmacopoeia 1990* edition, on December 3, 1990 with effective date on July 1, 1991.

This new edition of the pharmacopoeia is still published in two volumes containing 1751 monographs of substances and articles. 784 monographs including 509 monographs of Chinese traditional medicaments and 275 monographs of Chinese traditional patent preparations and single ingredient preparations are admitted to Volume I, while 967 monographs on chemicals, antibiotics, biological products and pharmaceutical preparations are admitted to Volume II. In comparison with the preceding edition, 80 new monographs are admitted and 3 monographs deleted from Volume I; 213 new monographs are admitted (including 5 monographs transferred from Volume I to Volume II) and 25 monographs deleted (3 from Volume I and 22 from Volume II). Appropriate changes have been made on titles of certain drug substances and articles as required. The headings "Action and use" and "Administration and dose" are changed to "Category" and "Dosage" respectively. "A Guide to Clinical Use of Drugs" was published as a companion volume to *the Chinese Pharmacopoeia* to serve as a guiding reference for medical and pharmaceutical practices. The infrared reference spectra are deleted from the Appendix with the publication of a separate volume—the Atlas of Infrared Spectra of Drugs.

The sixth Chinese Pharmacopoeia Commission was organized in 1991 with 168 members invited by the Ministry of Health, and Health Minister Chen Minzhang as its chairman. The first meeting of that Commission took place May 16-18 of the same year, attended by all members. In this meeting, the constitution of the Commission was further revised and the working plan for compilation and editing of *the Chinese Pharmacopoeia 1995* edition were discussed and substantial recommendations were made. A Standing Committee composed of the chairman, vice chairman and 11 other experts were set up with 13 subcommittees in respective specialized fields, namely, Chinese traditional medicine, Chinese traditional medicaments, Chinese traditional patent medicines, Modern medicine, Pharmacology, Chemicals I, II

and Ⅲ, Antibiotics, Biochemicals, Biological products, Radiopharmaceuticals and Nomenclature. The subcommittees then convened extended meetings in their specific fields, respectively, to work out the programs for revision of the Pharmacopoeia.

Drafts of the appendices of the 1995 edition were sent out in 1993 to relevant local organizations as a reference for the compilation and revision of the new edition. By July 1994 almost all local drafting had been finished and the subcommittees started organizing reviews. On Nov. 29, 1994 the drafts were discussed and further reviewed at an extended meeting of the Standing Committee, and then submitted to the Ministry of Health for approval. *The Chinese Pharmacopoeia* 1995 edition came into use as of April 1, 1996 as promulgated by the Ministry of Health.

There are 2375 monographs in that edition. 920 monographs, including 522 monographs of Chinese traditional crude drugs and of oils and fats, 398 monographs of Chinese traditional patent medicines and of preparations of single ingredient were admitted to Volume I. In Volume II, it was composed of 1455 monographs of chemicals, antibiotics, biochemicals, radiopharmaceuticals, biological products and some excipients. In comparison with the previous edition, 142 and 499 new admissions were admitted in Volume I, II respectively. English titles of drugs and preparation were adopted in Volume II and the Latin titles were deleted, while Chinese titles only use their official common names and no alternate names. The first Volume (1995 edition) of "Atlas of Infrared Spectra of Drugs and Medicines" was also compiled. "A Guide of Clinical Use of Drugs" was revised, and published together with the 1995 edition of *the Chinese Pharmacopoeia*. Ministry of Health approved that the "Indication" and "Dosage" in the later should be adopted as the basis for promotion of drug administration and management by competent authorities of drug management and drug manufacturing.

The sixth Chinese Pharmacopoeia Commission published the first and second addenda in 1992 and 1993, respectively. It also published "Commentary to Volume II of *the Chinese Pharmacopoeia* 1990 edition". "Selected Commentary to Volume I of *the Chinese Pharmacopoeia* 1990 edition", "Atlas of Traditional Chinese Medicines", "Atlas of Thin Layer Chromatography of Chinese Traditional Medicines" and "Adopted names of Chinese pharmaceutical products" as references in series relevant to pharmacopoeia. English version of *the Chinese Pharmacopoeia* 1990 edition was published in July 1993.

To strengthen standardization of drugs and medicines, the Ministry of Health decided, on May 21, 1993, that the Secretariat of the Chinese Pharmacopoeia Commission separated from the National Institute for the Control of Pharmaceutical and Biological Products and subordinated directly to the Ministry of Health. That was an important reform measure in the restructuring of the Commission.

Approved by the Ministry of Health, the seventh Chinese Pharmacopoeia Commission was set up in May 1996. The Ministry invited 204 members, including 18 honorary members, to form the Commission. Health Minister Chen Minzhang served as its Chairman. In September 1998 as approved by Document No. 32 (1998) of Central Office of Organization, the name of Pharmacopoeia Commission of the Ministry of Health was changed to the Chinese Pharmacopoeia Commission, and the administration of the Commission was transferred to the State Drug Administration (SDA). After change of the management system and passing away of Health Minister Chen Minzhang, the Standing Committee of the seventh Pharmacopoeia Commission decided to appoint chairman and vice chairmen of the Commission, as so stipulated in its Constitution in December 1999. Under that Commission there were 16 specialty

subcommittees; Chinese traditional medicine, Chinese traditional medicaments I, II, III, and IV, modern medicine, nomenclature, appendices, pharmaceutical preparations, pharmacology, chemicals I and II, antibiotics, biochemicals, radiopharmaceuticals and biological products.

The first meeting of the seventh Commission was held in 1996, at which the design plans for the 2000 edition of *the Chinese Pharmacopoeia* were endorsed. The guiding principles decided were that the Volume I should have special features in content and its quality should be largely improved, and the Volume II should reflect the combination of improvement and suitability to specific situations in China as well as a combination of advancement and characteristics. As scheduled in the plans, all subcommittees convened their own meetings and carried out their tasks starting from October 1996. By the end of 1997, all revision of the appendixes and general rules for drug and medicine preparations had been finished and sent to local drafting organizations for comments. A first draft was finalized at the end of 1998, and after reviews by related organizations in different parts of China; the 16 subcommittees further reviewed it by the end of October 1999. *The Chinese Pharmacopoeia 2000* edition was finally reviewed and passed by the seventh Chinese Pharmacopoeia Commission in December 1999, and submitted to the State Drug Administration for approval of publication. This edition was published in January 2000 and come into effect from July 1, 2000.

The 2000 edition contains a total of 2691 monographs, with 992 ones in Volume I and 1699 in Volume II. There are 399 new monographs and 562 revised ones in this edition. Appendixes have been considerably improved. There are 10 new and 31 revised appendixes in Volume I, and 27 new and 32 revised ones in Volume II. The Volume II has, for the first time, included 6 guidelines for Validation of Analytical Method Adopted in Pharmaceutical Quality Specification etc., which will play a role in the standardization and regulation of testing methods. Application of modern analytical techniques is further enhanced and stressed in this edition.

The seventh Chinese Pharmacopoeia Commission has also compiled the 1997 and the 1998 addendum of *the Chinese Pharmacopoeia 1995* edition, the “Adopted Names of Chinese Pharmaceutical Products (1998 Addendum)”, “Atlas of Infrared Spectra of Drugs and Medicines” (Volume II) and the third edition of “A Guide to Clinical Use of Drugs”. The English edition of *the Chinese Pharmacopoeia 1995* was published in 1997. To strengthen exchanges and cooperation, this Commission has decided to publish concurrently both Chinese and English versions of *the Chinese Pharmacopoeia 2000* edition.

“Dosage” and “Precaution” in preceding editions are too simple to reflect accurately the actual clinical use of drugs therefore deleted from this edition (Volume II) as so proposed in the design plans for the relevant contents of those two headings have been included in “A Guide to Clinical Use of Drugs”.

Approved by the State Drug Administration (redesigned to be State Food and Drug Administration in September 2003), the eighth Chinese Pharmacopoeia Commission was set up in October 2002. The State Drug Administration invited 312 experts as members of the Commission and did not appoint any honorary member. The Commissioner of the State Drug Administration, Mr. Zheng Xiaoyu served as its Chairman. The Standing Committee of the Commission was assigned as Executive Committee. The plenary session of the Chinese Pharmacopoeia Commission was authorized to examine and approve *the Chinese Pharmacopoeia* and the important items of the national specifications for pharmaceutical preparations. 24 subcommittees of specific duty and/or appointment are set up under the Commission. On the basis of previous Commission, 3 new subcommittees were established; namely ethnic medicines, microorganism, and packing materials and excipients; former subcommittee of biological products was expanded to 6 ones,

namely; blood products, viral vaccines, bacterial vaccines, somatic cell therapy and gene therapy, recombinant DNA products and diagnostic reagents for *in vivo test*.

The first meeting of the eighth Chinese Pharmacopoeia Commission and its Executive Committee took place in October 2002, approved “the design plans for the 2005 edition of *the Chinese Pharmacopoeia*”. The design plans clearly indicated that the *Pharmacopoeia* should adhere to guiding principles of “succession and development” and “theory combined with practice”; and editing principles of pharmacopoeia should be adhered to scientific, practical and specific basis. The meeting decided that *Requirements for Biologics of the People's Republic of China*, known as the *Chinese Requirements for Biologics* (CBR), would be integrated into the pharmacopoeia as its Volume III; “A Guide to Clinical Use of Traditional Chinese Patent Preparations” is to be compiled for the first time.

All designated subcommittee meetings have been convened since November 2002, to deal with the assigned duty recommended on the meeting in various aspects. By July 2003, the draft of appendices was accomplished at first and sent to relevant authorities and institutions for comments. Early in 2004, the first draft of appendices and text of pharmaceutical and biological products was basically ready and consecutively published on the website of the Chinese Pharmacopoeia Commission for 3 months, so as to get the feedbacks from various organizations and associations. Subcommittees, one after the other, convened meetings to review and revise the drafts from June to August, the Executive Committee of eighth Chinese Pharmacopoeia Commission endorsed *the Chinese Pharmacopoeia 2005* in September of the same year. In December 2004, the draft text was sent to the State Food and Drug Administration for approval and promulgation. *The Chinese Pharmacopoeia 2005* was published on January 2005 with effective date on July 1, 2005. The number of monographs in *the Chinese Pharmacopoeia 2005* is considerably increased. It contains up to 3214 monographs of drugs and other articles with 525 new admissions. Volume I contains 1146 monographs, with 154 new admissions and 453 revised; Volume II deals with 1967 monographs, with 328 new admissions and 522 revised; Volume III contains 101 monographs, with 44 new admissions and 57 revised. 9 monographs adopted in *the Chinese Pharmacopoeia 2000* are deleted in this edition. 123 monographs adopted in the *Requirements for Biologics of the People's Republic of China 2000* and in its *Supplement 2002* are not admitted in this edition.

The numbers of appendices in this edition are as follows; 98 monographs admitted in Volume I with 12 new admissions, 31 revised and 1 deleted; 137 monographs in Volume II with 13 new admissions, 65 revised and 1 deleted; 140 monographs in Volume III with 62 new admissions, 78 revised and 1 deleted. Appropriate monographs common to all three volumes are presented in each volume respectively in a harmonized and unified form.

Under the active leadership of the Chairman of the Chinese Pharmacopoeia Commission, the issue of pharmaceutical safety is emphasized particularly. In Volume I, atomic absorption spectrophotometry and inductively coupled plasma mass spectrometry are applied to determine the deleterious elements (lead, cadmium, mercury, arsenic and cuppers) and the limits of these elements have been stipulated; the guidelines of the safety test for the injections of traditional Chinese medicines is also added to the Volume I. In Volume II, the Test for Particulate Matter in Injections is applied to 126 injections intended for intravenous injections; the number of monographs adopting the Test for Bacterial Endotoxin reaches 112; the Determination of Residual Solvents includes the requirements of International Conference on

Harmonization (ICH) for residual solvents, and the test is required for 24 drug substances; In Volume II, the Guidelines for Analysis of Impurities in Drugs, Guidance for the Quality Control of Positron and Technetium [^{99m}Tc] Radiopharmaceutical Preparations are also admitted. In Volume III, new methods such as Determination of Reverse Transcriptase Activity and Test for Residual Aluminum Content in Human Albumin etc. are admitted, and some test methods are improved such as the test for residual bovine serum albumin and test for residual CHO cell protein, etc. On consideration of the status quo of medical industry and practical situation of drugs for clinical use, the requirements set forth in Detailed Regulations for Clarity Test and Criteria formerly issued by the Ministry of Health are replaced by the method for Determination of Visible Particles in this edition so as to enhance the safety of pharmaceuticals including injections.

The Chinese Pharmacopoeia 2005 attaches great importance to the consistent principle of environmental protection, therefore the harmful solvents, such as benzene etc. used in pharmaceuticals should be substituted by other solvents as far as possible.

According to theory of Traditional Chinese Medicine—diagnosis and treatment based on an overall analysis of the illness and the patient's condition, the *Indications* under the Chinese patent preparations have been scientifically standardized so as to provide the assurance of understanding the *Indications* precisely and making use of drugs rationally, and to facilitate the healthy development of traditional Chinese medicine.

Volume III of this edition is originated from *CBR*. Six editions of the *CBR* have been promulgated for implementation since 1951, i. e. the edition 1951 and its addendum 1952, and the edition 1959, 1979, 1990 and 1993 (for diagnostic products), 1995 and 2000 and its supplement were published respectively. The English version of *CBR 2000* was published for the first time in 2002.

The eighth Chinese Pharmacopoeia Commission also has completed the Addendum 2002 and Addendum 2004 of *the Chinese Pharmacopoeia 2000*, the Adopted Names of Chinese Pharmaceutical Products (2005 edition), Atlas of Infrared Spectra of Drugs and Medicines (third volume) and “Guide to Clinical Use of Drugs” (the first edition for Chinese traditional patent preparations and the fourth edition for chemicals).

The English version of *the Chinese Pharmacopoeia 2005* was completed in 2005. In order to enhance the international cooperation and communication, the Chinese Pharmacopoeia Commission has organized the first “China-USA joint forum on Pharmacopoeia”.

Aiming at the strengthening and improving the efficiency and level of national standard work, the Secretariat of the Chinese Pharmacopoeia Commission has completed the construction of office automation and realized standards enacted the Chinese for computer network retrieval and statistical analysis.

New Monographs Included in Volume III (2005)

Biologics for Prophylaxis

Dysentery Vaccine (Live) of *S. flexneri* and *S. sonnei*, Oral
Rubella Vaccine (Rabbit Kidney Cell), Live
Measles and Mumps Combined Vaccine, Live
Hepatitis A (Live) Vaccine, Freeze-dried
Influenza Vaccine (Whole Virion), Inactivated

Biologics for Therapeutic Use

Human Prothrombin Complex
Anti-human T Lymphocyte Porcine Immunoglobulin
Anti-human T Lymphocyte Rabbit Immunoglobulin
Recombinant Human Interferon α 2a for Injection (Yeast)
Recombinant Human Interferon α 2b for Injection (*P. putida*)
Recombinant Human Interferon α 2b Injection (*P. putida*)
Recombinant Bovine Basic Fibroblast Growth Factor for External Use, Liquid
Recombinant Bovine Basic Fibroblast Growth Factor for External Use
Recombinant Bovine Basic Fibroblast Growth Factor Eye Drops
Recombinant Human Epidermal Growth Factor for External Use
Recombinant Human Epidermal Growth Factor Derivative for External Use, Liquid
Mouse Monoclonal Antibody against Human CD3 Antigen of T Lymphocyte for Injection

Others

1. Monographs of 17 biologics with both liquid and freeze-dried forms are presented;
2. Three monographs of Poliomyelitis (Live) Vaccine are included, according to the different animal cell substrates used for production;
3. Two monographs of Rabies Vaccine for Human Use are included, according to the different animal cell substrates used for production;
4. Two monographs of Rubella Live Vaccine are included, according to the different animal cell substrates used for production;
5. Four monographs of Antitoxins are included, according to the different kinds of antitoxins;
6. Four monographs of Snake Antivenins are included, according to the different kinds of snake antivenins.

Monographs Deleted from the Previous Editions

Biologics for Prophylaxis

Tick-borne Encephalitis Vaccine, Inactivated
Haemorrhagic Fever With Renal Syndrome (Type I) Purified Vaccine
Yellow Fever Vaccine, Live
Cholera Toxoid and Whole Cell Vaccine, Adsorbed
Live Rotavirus Vaccine, Oral
Tracheitis Vaccine
Furunculosis Vaccine
Tracheitis Bacteriolysate Vaccine

Biologics for Therapeutic Use

BCG Polysaccharide and Nucleic Acid Preparation
Brucella Vaccine for Therapeutic Use
Corynebacterium Parvum Preparation
Group A Streptococcus Preparation
Nocardia Rubra Cell Wall Skeleton preparation
Pseudomonas Aeruginosa Preparation
Bacillus Subtilis Preparation for Spraying
Pseudomonas Preparation
Placenta Derived Anti-HBV Transfer Factor Injection
Staphylococcin Injection
Anti-HBV Transfer Factor Injection
Albumin Prepared from Human Placenta
Human Histamine Immunoglobulin
Extract of Human Placenta
Lipid Polysaccharide Prepared from Human Placenta

Probiotics

Live Bifidobacterium Preparation, Oral
Live Combined Bifidobacterium, Lactobacillus and Enterococcus Preparation, Oral
Live Combined Bifidobacterium, Lactobacillus and *Streptococcus thermophilus* Tablets, Oral
Live *Bacillus licheniformis* Preparation, Oral
Live *Bacillus cereus* Preparation, Oral

Diagnostic Reagents for *in vitro* Test (2000 edition)

Bacterial Suspensions of Typhoid-paratyphoid and Proteus OX19, OX2 and OXK
Diagnostic Sera for Salmonella

Diagnostic Sera for Shigella
 Diagnostic Sera for Entero-pathogenic *Escherichia coli*
 Diagnostic Sera for Leptospira
 Diagnostic Sera for *Neisseria gonorrhoeae*
 Diagnostic Sera for Grouping Reagents (Monoclonal Antibody) for *Neisseria meningitidis* A, B and C
 Diagnostic Sera for Group O1 Vibrio Cholera
 Diagnostic Reagent of Diphtheria Antibody (Hemagglutination)
 Diagnostic Reagent of Tetanus Antibody (Hemagglutination)
 Diagnostic Sera for *Yersinia pestis*
 Diagnostic Reagent for F1 Antibody of *Yersinia pestis* (Hemagglutination)
 Phage for Diagnosis of *Yersinia pestis*
 Diagnostic Sera for *Bacillus anthracis*
 Phage for Diagnosis of *Bacillus anthracis*
 Diagnostic Sera for Botulinum Toxin
 Syphilis Rapid Plasma Reagin (RPR)
 Syphilis Toluidine Red Untreated Serum Test (TRUST)
 Diagnostic Kit for Antibody to Japanese Encephalitis (Hemagglutination Inhibition)
 Diagnostic Kit for IgM Antibody to Hepatitis A Virus (ELISA)
 Diagnostic Kit for Antibody to Hepatitis A Virus (ELISA)
 Diagnostic Kit for Hepatitis B Surface Antigen (RIA)
 Diagnostic Kit for Antibody to Hepatitis B Surface Antigen (RIA)
 Diagnostic Kit for Antibody to Hepatitis B Core Antigen (RIA)
 Diagnostic Kit for Hepatitis B e Antigen (RIA)
 Diagnostic Kit for Antibody to Hepatitis B e Antigen (RIA)
 Diagnostic Kit for Hepatitis B Surface Antigen (ELISA)
 Diagnostic Kit for Antibody to Hepatitis B Surface Antigen (ELISA)
 Diagnostic Kit for Antibody to Hepatitis B Core Antigen (ELISA)
 Diagnostic Kit for Hepatitis B e Antigen (ELISA)
 Diagnostic Kit for Antibody to Hepatitis B e Antigen (ELISA)
 Diagnostic Kit for Antibody to Hepatitis C Virus (ELISA)
 Diagnostic Kit for Antibody to Human Immunodeficiency Virus (ELISA)
 Anti-A and Anti-B Blood Grouping Reagents (Human Sera)
 Anti-A and Anti-B Blood Grouping Reagents (Horse Sera)
 Anti-A and Anti-B Blood Grouping Reagents (Monoclonal Antibody)
 Human Lymphocyte Subgroup Typing Reagent (Monoclonal Antibody)
 Plasma Protein Detection Reagent
 Diagnostic Sera for Human Immunoglobulin G, A, M, D and E
 Diagnostic Kit for Tissue Plasminogen Activator (t-PA) (ELISA)
 Diagnostic Kit for Alpha-feto-protein (ELISA)
 Diagnostic Kit for Carcinoembryonic Antigen (CEA) (ELISA)
 Diagnostic Kit for Human Chorionic Gonadotrophin (HCG) (Colloidal Gold)
 Essentials for Production and Control of Haemolysin
 Essentials for Production and Control of Total Complement
 Essentials for Production and Control of Anti-IgG Serum
 Essentials for Production and Control of Horseradish Peroxidase (HRP)

Labeled Immunoglobulin Conjugate

Essentials for Production and Control of Biotin Labeled IgG

Essentials for Production and Control of Fluorescence Labeled Immunoglobulins

Essentials for Production and Control of Anti-Mouse IgG and Its Subgroup Sera

Essentials for Production and Control of Calciferous Thromboplastin

Essentials for Production and Control of *Escherichia coli* Endotoxin

Essentials for Production and Control of Immunodiffusion Plate for Human Immunoglobulin G, A, M and Complement C3 and C Reactive Protein

Essentials for Production and Control of Quality Control Sera

Essentials for Production and Control of Antigen Used for Glass Slide Test in Venereal Disease Research Laboratory (VDRL)

Essentials for Production and Control of *Yersinia pestis* F1 Antigen

Essentials for Production and Control of Antisera to *Yersinia pestis* Phage

Essentials for Production and Control of *Bacillus anthracis* Antigen

Diagnostic Reagents for *in vitro* Test (2002 Supplement)

Diagnostic Kit for Antibody to *H. pylori* Urease (ELISA)

Diagnostic Reagent for Antibody to Schistosoma Egg (Hemagglutination)

Diagnostic Kit for Antibody to Schistosoma Egg (ELISA)

Diagnostic Kit for Circulating Antigen of Cysticercus Cellulosa (ELISA)

Morphine Diagnostic Strips (Colloidal Gold)

Diagnostic Kit for Antibody to Treponema Pallidum (Indirect-ELISA)

Diagnostic Kit for Phenylketonuria

Diagnostic Kit for Luteinizing Hormone (Colloidal Gold)

Diagnostic Kit for Fecal Occult Blood (Colloidal Gold)

Diagnostic Kit for Hepatitis B PreS₂ Antigen (ELISA)

Diagnostic Kit for Hepatitis B PreS₂ Antibody (ELISA)

Factor IX Deficiency Plasma

Diagnostic Reagents for *in vitro* Test (2000 Interim)

Diagnostic Erythrocytes Sensitized with Groups of *N. meningitis* and Mixed Antigen, Freeze-dried

Diagnostic Sera for *Pseudomonas aeruginosa*

Diagnostic Sera for Grouping of *Bacillus cereus*, Freeze-dried

Diagnostic Sera Labeled with SPA for Grouping Reagents of *Bacillus cereus*

Diagnostic Sera for Brucella

Bacterial Suspensions of Brucella Used for Tube Agglutination

Bacterial Suspensions of Brucella Used for Glass Slide Agglutination

Diagnostic Kit for Treponema Pallidum Haemagglutination (TPHA), Freeze-dried

Hemagglutinin of Japanese Encephalitis Virus, Freeze-dried

Diagnostic Sera for Japanese Encephalitis Virus

Hemagglutinin of Measles Virus

Hemagglutinin of Rubella Virus

Diagnostic Sera for Poliomyelitis virus, Freeze-dried

Diagnostic Kit for Antibody to Human Immunodeficiency Virus, Type I (Western Blot)

Diagnostic Kit for IgA to EB Virus (ELISA)

Diagnostic Kit for Rotavirus Virus (ELISA)

Diagnostic Erythrocytes for Haemorrhagic Fever

Diagnostic Sera for Anti-human Immunoglobulin Heavy Chain, Type γ , α and μ

Diagnostic Sera for Anti-human Immunoglobulin Light Chain, Type κ , λ

Diagnostic Kit for Human Ig E (ELISA)

Diagnostic Erythrocytes of Equine Anti-human Ig E, Freeze-dried

Diagnostic Erythrocytes of Alpha-feto-protein, Freeze-dried

Diagnostic Sera for Human Complement C3, Freeze-dried

Diagnostic Reagents for Mouse Monoclonal Antibody PAP for Immune Histochemistry Staining, Freeze-dried

Diagnostic Reagent for Human Chorionic Gonadotrophin (HCG) (Latex Agglutination Inhibition Test)

Appendices Added, Revised and Deleted in Volume III (2005)

Appendices Added in Volume III (2005)

Appendix I General Requirements for Preparations

- Appendix I A Injections
- Appendix I B Suppositories
- Appendix I C Eye Preparations
- Appendix I D Liquids for External Application
- Appendix I E Tablets
- Appendix I F Capsules
- Appendix I G Ointments, Emulsions
- Appendix I H Sprays
- Appendix I J Granules
- Appendix I K Powders
- Appendix I L Nasal Preparations
- Appendix I M Gels

Appendix II Spectrophotometry

- II A Ultraviolet-visible Spectrophotometry
- II B Atomic Absorption Spectrophotometry
- II C Fluorometry
- II D Flame Photometry

Appendix III Chromatography

- III A Paper Chromatography
- III B High Performance Liquid Chromatography
- III C Gas Chromatography
- III D Size Exclusion Chromatography

Appendix IV Electrophoresis

- IV B Agarose Electrophoresis

Appendix V

- V B Test for Visible Particles
- V C Test for Disintegration

- V D Disintegration Test for Suppositories and Vaginal Tablets
- V E Test for Tablet Friability
- V F Test for Minimum Fill
- V G Determination of Particle Size
- V H Determination of Osmolality

Appendix VI

- VI B Determination of Protein Content
Method 3 Biuret Method
- VI D Determination of Residual Ethanol Content
- VI E Determination of Free Histamine Phosphate Content in Human Histamine Immunoglobulin

Appendix VII

- VII K Determination of Residual Aluminium Content in Human Albumin
- VII L Determination of Loss on Drying

Appendix VIII

- VIII G Determination of Molecular Size for Group A Meningococcal Polysaccharide
Method 2 Instrumental Method
- VIII H Determination of Molecular Size for Typhoid Vi Polysaccharide

Appendix IX

- IX D Determination of Residual Host Bacterial Protein (Pseudomonas)
- IX E Determination of Residual Host Yeast Protein
- IX G Test for Losing Rate of Plasmid
- IX L Determination of Residual Murine IgG
- IX M Test for Reverse Transcriptase Activity
- IX N Test for Human Thrombin Activity
- IX O Test for Activated Coagulation Factor Activity
- IX P Determination of Heparin Content
- IX Q Determination of Human Erythrocyte Antibody
- IX R Determination of Human Platelet Antibody

Appendix X

- X G Biological Activity Test for Recombinant Bovine Basic Fibroblast Growth Factor
- X H Biological Activity Test for Recombinant Epidermal Growth Factor
- X J Potency Test for Human Coagulation Factor II
- X K Potency Test for Human Coagulation Factor VII
- X L Potency Test for Human Coagulation Factor IX
- X M Potency Test for Human Coagulation Factor X
- X P Test for Fc Function in Human Immunoglobulin
- X Q Potency Test for Anti-human T Lymphocyte Immunoglobulin (E-rosette Formation-inhibition Test)
- X R Potency Test for Anti-human T Lymphocyte Immunoglobulin (Lymphocytotoxicity Test)

Appendix XI

XI D Determination of Flocculation Unit of Toxiod

Appendix XII

XII G Microbial Limit Test

Appendix XIII B

XIII B Test Requirements of Microbes for Laboratory Animals

XIII C Test Requirements of Parasites for Laboratory Animals

XIII D Test Requirements for Calf Serum

Appendix XIV

XIV Culture Media for Biochemical Reactions of Bacteria and Test Method

Appendix XV

XV Sterilization

Appendix XVI

XVI Names, Symbols and Atomic Weights

Appendices Revised in Volume III (2005)

Appendix IV

IV A Cellulose Acetate Film Electrophoresis

IV C SDS-Polyacrylamide Gel Electrophoresis

IV D Isoelectric Focusing Electrophoresis

Appendix V

Appendix V A Determination of pH Value

Appendix VI

VI A Determination of Nitrogen

VI B Determination of Protein Content

Method 1 Keldahl Method

Method 2 Lowry Method

VI C Determination of Sialic Acid Content

VI F Determination of O-Acetyl Content

VI G Determination of Residual Polyethylene Glycol Content

VI H Determination of Residual Polysorbate 80 Content

VI I Determination of Residual Glutaraldehyde Content

VI J Determination of Tributylphosphate Content

VI K Determination of Sodium Caprylate Content

VI L Determination of Free Formaldehyde Content

VI M Determination of Phenol Content

VI N Determination of Metacresol Content

- VI O Determination of Trichloromethane Content
- VI P Determination of Saccharides and Sugar Alcohol Content in Human Blood Products
- VI Q Determination of Polymer Content in Human Albumin
- VI R Determination of IgG Monomer and Dimer in Human Immunoglobulins

Appendix VII

- VII A Determination of Phosphorus Content
- VII B Determination of Thimerosal Content
- VII C Determination of Ammonium Sulfate Content
- VII D Determination of Moisture Content
- VII E Determination of Sodium Bisulfite Content
- VII F Determination of Aluminium Hydroxide (or Aluminium Phosphate) Content
- VII G Determination of Sodium Chloride Content
- VII H Determination of Citrate Content
 - Method 1 Colourimetric Method
 - Method 2 HPLC Method
- VII I Determination of Potassium Content
- VII J Determination of Sodium Content
- VII M Determination of Total Solid

Appendix VIII

- VIII A Immunoblot
- VIII B Immunodot
- VIII C Double Immunodiffusion
- VIII D Immunoelectrophoresis
- VIII E Peptide Mapping
 - Method 1 Trypsin Cleavage-Reverse Phase HPLC Method
 - Method 2 Cyanogen Bromide Cleavage Method
- VIII F Determination of F (ab)₂ Content in Antitoxin
- VIII G Determination of Molecular Size for Group A Meningococcal Polysaccharide
 - Method 1 Phosphorous Determination Method

Appendix IX

- IX A Determination of Residual Antibiotics
- IX B Determination of Residual Extraneous DNA
- IX C Determination of Residual Host Bacterial Protein (*E. coli*)
- IX F Determination of Prekallikrein Activator Content
- IX H Test for Nucleotide Sequence of SV40
- IX I Test for Blood Group A-like Substance
- IX J Test for Anti-A and Anti-B Hemoagglutinins
- IX K Test for Anticomplement Activity

Appendix X

- X A *In vitro* Test for Relative Potency of Recombinant Hepatitis B Vaccine (Yeast)
- X B *In vivo* Test for Biological Activity of Recombinant Erythropoietin

- X C Biological Activity Test for Interferon
- X D Biological Activity Test for Recombinant Human Interleukin-2
- X E Biological Activity Test for Recombinant Human Granulocyte Colony-stimulating Factor
- X F Biological Activity Test for Recombinant Human Granulocyte/Macrophage Colony-stimulating Factor
- X I Biological Activity Test for Recombinant Streptokinase
- X N Potency Test for Human Coagulation Factor VIII
- X O Potency Test for Diphtheria Antibody in Human Immunoglobulin

Appendix XI

- XI A Potency Test for Rabies Vaccine for Human Use
- XI B Potency Test for Adsorbed Tetanus Vaccine
- XI C Potency Test for Adsorbed Diphtheria Vaccine
 - Method 1 Toxin Challenge Method in Guinea Pig
 - Method 2 Mouse-Vero Cell Antibody Titration Method
- XI E Potency Test for Diphtheria Antitoxin
- XI F Potency Test for Tetanus Antitoxin
- XI G Potency Test for Gas-gangrene Antitoxin
- XI H Potency Test for Botulinum Antitoxin
- XI I Potency Test for Snake Antivenins
- XI J Potency Test for Rabies Antiserum
- XI K Determination of IgG Content
- XI L Test for Neurovirulence in Monkeys

Appendix XII

- XII A Sterility Test
- XII B Test for Mycoplasma
- XII C Test for Adventitious Virus
- XII D Pyrogen Test
- XII E Test for Bacterial Endotoxin
- XII F Test for Abnormal Toxicity
- XII H Test for Murine Virus

Appendix XIII

- XIII A Test Requirements for SPF Chicken Embryos

Deleted in Volume III (2005)

Determination of Ribose Content

Revised Titles in Volume III (2005)

Biologics for Prophylaxis

- Dysentery Vaccine (Live) of *S. flexneriza* and *S. sonnei*, Oral
(previous title: Live *Sh. Flexneri 2a* and *Sh. Sonnei* Combined Vaccine)
- Rabies Vaccine (Vero Cell) for Human Use
- Rabies Vaccine (Vero Cell) for Human Use, Freeze-dried
- Rabies Vaccine (Hamster Kidney cell) for Human Use
(previous title: Rabies Purified Vaccine for Human Use)
- Rubella Vaccine (Human Diploid Cell), Live
- Rubella Vaccine (Rabbit Kidney Cell), Live
(previous title: Rubella Vaccine, Live)
- Poliomyelitis Vaccine in Dragee Candy (Human Diploid Cell), Live
- Poliomyelitis (Live) Vaccine (Monkey Kidney Cell), Oral
- Poliomyelitis Vaccine in Dragee Candy (Monkey Kidney Cell), Live
(previous title: Poliomyelitis Vaccine, Oral)

Biologics for Therapeutic Use

- Human Hepatitis B Immunoglobulin
- Human Hepatitis B Immunoglobulin, Freeze-dried
(previous title: Human Hepatitis B Immunoglobulin)
- Human Rabies Immunoglobulin
- Human Rabies Immunoglobulin, Freeze-dried
(previous title: Human Rabies Immunoglobulin)
- Human Tetanus Immunoglobulin
- Human Tetanus Immunoglobulin, Freeze-dried
(previous title: Human Tetanus Immunoglobulin)
- Human Immunoglobulin (pH 4) for Intravenous Injection
- Human Immunoglobulin (pH 4) for Intravenous Injection, Freeze-dried
(previous title: Human Immunoglobulin (pH 4) for Intravenous Injection)
- Human Immunoglobulin for Intravenous Injection
(previous title: Human Immunoglobulin for Intravenous Injection, Freeze-dried)
- Human Coagulation Factor VIII
(previous title: Human Coagulation Factor VIII, Freeze-dried)
- Human Fibrinogen
(previous title: Human Fibrinogen, Freeze-dried)
- Human Prothrombin Complex
(previous title: Human Prothrombin Complex, Freeze-dried)
- Anti-human T Lymphocyte Porcine Immunoglobulin

(previous title: Anti-human T Lymphocyte Immunoglobulin)
Anti-human T Lymphocyte Rabbit Immunoglobulin
(previous title: Rabbit Anti-human T Lymphocyte Immunoglobulin)
Mouse Monoclonal Antibody against Human CD3 Antigen of T Lymphocyte for Injection
(previous title: Monoclonal Antibody of Mouse Anti-human CD3 Antigen of T Lymphocyte)
Botulinum Toxin Type A for Injection
(previous title: Botulinum Toxin Type A for Therapeutic Use)
Recombinant Human Interferon α 1b for Injection
Recombinant Human Interferon α 1b Injection
(previous title: Recombinant Human Interferon α 1b)
Recombinant Human Interferon α 2a for Injection
Recombinant Human Interferon α 2a Injection
(previous title: Recombinant Human Interferon α 2a)
Recombinant Human Interferon α 2a for Injection (Yeast)
[previous title: Recombinant Human Interferon α 2a (Yeast)]
Recombinant Human Interferon α 2a Vaginal Suppository
(previous title: Recombinant Human Interferon α 2a in Suppository Form)
Recombinant Human Interferon α 2b for Injection
Recombinant Human Interferon α 2b Injection
(previous title: Recombinant Human Interferon α 2b)
Recombinant Human Interferon γ for Injection
(previous title: Recombinant Human Interferon γ)
Recombinant Human Interleukin-2 for Injection
(previous title: Recombinant Human Interleukin-2)
Recombinant Human Erythropoietin For Injection (CHO Cell)
Recombinant Human Erythropoietin Injection (CHO Cell)
(previous title: Recombinant Human Erythropoietin)
Recombinant Human Granulocyte Colony-stimulating Factor Injection
(previous title: Recombinant Human Granulocyte Colony-stimulating Factor)
Recombinant Human Granulocyte/Macrophage Colony-stimulating Factor for Injection
(previous title: Recombinant Human Granulocyte/Macrophage Colony-stimulating Factor)
Recombinant Streptokinase for Injection
(previous title: Recombinant Streptokinase)
Recombinant Bovine Basic Fibroblast Growth Factor for External Use, Liquid
(previous title: Recombinant Bovine Basic Fibroblast Growth Factor for External Use)
Recombinant Human Epidermal Growth Factor Derivative for External Use, Liquid
(previous title: Recombinant Human Epidermal Growth Factor Derivative for External Use)
Diphtheria Antitoxin
Diphtheria Antitoxin, Freeze-dried
Tetanus Antitoxins
Tetanus Antitoxins, Freeze-dried
Gas-gangrene Antitoxin (Mixed),
Gas-gangrene Antitoxin (Mixed), Freeze-dried
Botulinum Antitoxin
Botulinum Antitoxin, Freeze-dried

(previous title: Antitoxin)

Agkistrodon halys Antivenin, Equine

Agkistrodon acutus Antivenin, Equine

Bungarus multicinctus Antivenin, Equine

Naja naja (*atra*) Antivenin, Equine

(previous title: Snake Antivenins)

General Notices

The Volume Ⅲ of the Pharmacopoeia of the People's Republic of China, known as Volume Ⅲ of the Chinese Pharmacopoeia in abbreviation, serves as official technical standards for supervision and administration of quality of biological products in China.

Once the Pharmacopoeia of the People's Republic of China is promulgated for implementation by the National Regulatory Authority (NRA), the previous national standards of the same kinds of products shall become invalid. Unless otherwise stated, the Chinese Pharmacopoeia stated in this edition indicates the current one.

The General Notices function as a basic guideline for the interpretation and application of the standards in Volume Ⅲ of the Chinese Pharmacopoeia for the production and testing of biological products. The General Notices provide in summary form regulations for the common issues in individual monographs or appendices related in the production, quality control and other specifications to obviate the need to repeat the requirements and other descriptions that are pertinent to numerous instances. The requirements set forth in the General Notices are official in the Pharmacopoeia.

The expression of "unless otherwise stated" is adopted indicating that appropriate requirement is admitted in the related monograph wherever it is not conform to that specified in General Notices, General Requirements or Appendices.

The expression of "keeping to the approved production process, the seed/strain or medium used for production", or "keeping to the approved validity period of a product" is adopted in individual monographs indicating that the production process, the seed/strain or medium used for production, or validity period of a product has been approved by the NRA.

A formulated preparation must comply throughout its assigned period of validity.

Title and arrangements

1. The adopted Chinese names of biological products admitted in this edition are named according to the principles for nomenclature of *Chinese Approved Drug Names of Pharmaceuticals*. The adopted Chinese names of biological products in the Chinese Pharmacopoeia serve as official names. An English name shall be given accordingly referring to the WHO requirements or the international practice. International Nonproprietary Names (INN) adopted for pharmaceuticals can also be used.
2. This edition is composed of three parts: general requirements, monographs and appendices. The products admitted in the monographs of this edition range from vaccines (including viral and bacterial vaccines, and combined vaccines), antitoxins and antisera, blood products, recombinant DNA products, diagnostic reagents for *in vivo* test to other biologics.

The requirements in each monograph are stated with respect to the following items: Titles in Chinese and English and Chinese phonetic alphabet; descriptive definition; basic requirements; manufacturing; control tests on bulk, final bulk and final product; storage, shipping and validity period. Package inserts of the products for prophylaxis are also included in monographs.

Manufacturing

3. Facilities and production management

The Chinese Good Manufacturing Practice for Pharmaceutical Product shall apply.

(1) For *Bacillus anthracis*, *Clostridium botulinum* and *Clostridium tetani*, strictly dedicated facilities shall be utilized for each individual product.

(2) Dedicated facilities and equipment shall be used for the manufacture of medicinal products derived from human blood or plasma. No products derived from animal proteins shall be handled in the facilities.

(3) BCG vaccine shall be produced in a dedicated facility. The production facilities for BCG and tuberculin shall be strictly separated and the equipment shall be the dedicated.

4. Bacterial and viral seeds/strains and cells derived from human or animal, or strains and cells constructed by recombinant DNA techniques used directly in production and quality control shall be subject to the approval of the NRA.

5. Source and subsidiary materials

The quality of subsidiary materials and the source materials used in production shall comply with the specifications set forth in Volume II of the Chinese Pharmacopoeia. For those materials not compiled in the Pharmacopoeia, the criteria meeting the standards for medicinal use shall be established. The subsidiary materials used shall be subject to the approval by the NRA.

The culture medium used in production shall be free from any substances that may cause adverse reactions in humans.

6. Water and apparatus for production

The source water for production shall meet the national standards of potable water, purified water and water for injection shall meet the standards of the current Chinese Pharmacopoeia. The preparation, storage, distribution and usage of the water for production shall comply with the requirements set forth in the Chinese GMP for Pharmaceutical Products.

The apparatus made of metal or glass or other materials used directly in production shall be washed and cleaned thoroughly and sterilized.

7. Unless otherwise stated, penicillin or β -lactam antibiotics must not be used at any stage in the production process. Non-asbestos filters shall be used for filtration.

8. Animals used in production and control tests

(1) Cell cultures used for the preparation of live vaccines for injection shall come from the animals which shall satisfy the standards for clean or SPF animals. Cell cultures used for the preparation of oral and inactivated vaccines shall come from healthy animals drawn from a uniform stock, and shall be tested for the specific viruses associated with the animals used. In-breed mice shall be used.

(2) Serum of bovine origin used for cell cultures must come from herds certified to be free of bovine spongiform encephalopathy and the quality of the serum shall comply with the related requirements of the Pharmacopoeia.

(3) Trypsin used for preparing cell cultures shall be shown free from contamination of adventitious or endogenous agents.

(4) The flocks from which the chick embryos or embryo cells are provided for production shall be

specific pathogen-free animals, unless otherwise stated.

(5) Horses used for production shall comply with the Requirements for Quarantine and Immunization of Horses Used for Preparation of Immune Sera.

(6) Animals used for quality control shall, unless otherwise stated, satisfy the standards for clean or SPF animals. Chemical and physical methods or cytological methods shall be used as much as possible instead of animal method for testing of biological products so as to reduce the use of animals in tests.

9. Quality control shall consist of safety and efficacy, and shall be of controllability. The substances to be tested as stated in monograph refer to those need to be controlled, during production and storage as required. When a change of production process is made, testing items and standards shall be modified accordingly.
10. Testing method shall be available for individual quality standard of product. The testing method shall be feasible and reproducible with a definite result for evaluation. For a newly established method, result verification shall be demonstrated by at least three independent laboratories. The precision of the testing result shall be expressed by the same number of places of significant digits as that of the value given in the technical requirements.

Precision and accuracy

11. The accuracy for sampling quantity of test material and the precision for testing

(1) Weight and measure of substances being examined and reagents being used are expressed in Arabic figures. The required precision or accuracy is expressed by the significant decimals. For example, the measurement of "0.1 g" by weight refers to that 0.06-0.14 g of the substance may be weighed, for "0.2 g", 1.5-2.5 g of the substance may be weighed, for "2.0 g" and "2.00 g", 1.95-2.05 g and 1.995-2.005 g of substances may be weighed, respectively.

Weigh accurately indicates that the measurement shall be made to an accuracy of 0.1%; *weigh* indicates an accuracy shall be made to 1%; *measure accurately* indicates that the accuracy of the volume being measured complies with the national standard of pipette being used for the measurement of the required volume. *Measure* indicates that the measuring cylinder or other measuring apparatus being used complies with the requirements for the measurement of volume to the significant value or decimal. The word *about* states that the measuring quantity should not exceed $\pm 10\%$ of the specified quantity.

(2) *Constant weight*, unless otherwise stated, used in relation to the process of drying or the process of ignition means that two consecutive weighings do not differ by more than 0.3 mg. The second and the subsequent weighings shall be made after an additional hour of drying each time under specified conditions, the second weighing of the substance being made after an additional 30 minutes of ignition.

(3) *Blank test* refers to a test carried out in the similar manner without the substance being examined or using the same amount of solvent instead of the solution being tested. The statement of *to make any necessary correction of the result with a blank test* refers to that the result is calculated by subtracting number of milliliters of titrant used in blank test from that consumed in assay of the substance being examined.

(4) *Diagnostic reagents* used in the control tests shall be approved by the NRA, unless otherwise stated.

(5) *Water* used in tests and assays, unless otherwise stated, refers to purified water. *Water* used for measurement of acidity or alkalinity refers to that of freshly boiled and cooled to room temperature.

(6) *Temperature* for a test refers to a room temperature whenever the temperature is not stated. In case that the temperature variation influences significantly to the testing result, it shall be tested at a temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, unless otherwise stated.

Testing methods and limitation

12. The assays and tests described in this Pharmacopoeia are the official methods with which the products should be tested. If other methods are to be used, comparison study on the alternative and stated methods shall be performed to demonstrate that the method to be adopted will give a result of equivalent accuracy. In the event of doubt or dispute, the methods of this Pharmacopoeia are alone authoritative.
13. Where limits and various degrees of purity as well as the amount of weight or filling and their permissible deviations stated in the standards are expressed numerically herein, the upper and lower limits themselves and all intermediate values should be included. The limits expressed in monograph definitions and tests, regardless of whether the values are expressed as percentages or as absolute numbers, are considered significant to the last digit shown.

Analytical results observed in the experiment should be compared with the stated limits to determine whether there is conformance with the test requirements. The observed or calculated values usually contain one more significant figures than there are in the stated limit, and an observed or calculated result should be rounded off to the number of places that is in agreement with the limit expression by the rule of rounding off.

The rule of rounding off:

When rounding off is required for the analytic results obtained in testing or calculation, the standards for rounding off described in the * GB30101-93 shall apply, which can be summarized as follows.

Rule of rounding off	Example	
	Unrounded value	Rounded result (if one digit in the decimal place is retained)
The last figure is unchanged when the part rejected is smaller than 5 (less than a half-unit).	14.2432	14.2
The last figure is increased by one when the part rejected is greater than 5 (exceeds one half-unit).	26.4643	26.5
If there is a figure following 5, the preceding digit is increased by one.	11.0512	11.1
In case that it is a zero following 5, if it is an odd number before 5, the preceding digit is increased by one; if it is an even number before 5, the preceding digit is unchanged.	1.3500 1.4500 1.0500	1.4 1.4 1.0 (zero is considered as an even number.)
No matter how many digits should be rejected, it shall be done once.	1.54546	1.5 (not 1.5455→1.546→1.55→1.6)

* GB 30101-93; China National Standard 30101-93

Standards, references and reference substances

14. National biological standards and references serve as the standard substances used for determination of potency, activity or content of biological products or used for identification of and characterization of biologics. The preparation and calibration shall comply with the Requirements for Preparation and Calibration of National Standard Substances for Biologics of the General Requirements of this Pharmacopoeia, and the standard substances shall be distributed by the institutions designated by the NRA. In-house standards or references shall be calibrated against the national standard substances before use.

Reference substances refer to the specific reference substances used for biological assays of biological products, while chemical reference substances (CRS) refer to the specific reference substances used for chemical testing. Reference substances shall be checked and verified by the NCL. Unless otherwise stated, reference substances shall be calculated as that of dried materials (or anhydrous materials) before use.

Units of measurement

15. Measuring instruments used in tests or assays shall comply with the relevant requirements promulgated by the National Bureau of Technical Supervision.
16. Units of measurement adopted in the Pharmacopoeia

(1) The official names and symbols of units of measurement are listed as follows.

Length: m (meter), dm (decimeter), cm (centimeter), mm (millimeter), μm (micrometer), nm (nanometer);

Volume: L (liter), ml (milliliter), μl (microliter);

Mass weight: kg (kilogram), g (gram), mg (milligram), μg (microgram), ng (nanogram), pg (picogram);

Pressure: MPa (megapascal), kPa (kilopascal), Pa (pascal),

(2) In this edition of Pharmacopoeia, the strengths or concentrations of the volumetric solutions and test solutions are expressed in terms of mol/L or mmol/L, and in term of "XXX solution (YYY mol/L)" for accurate standardization. They are expressed in term of "YYY mol/L XXX solution" for other purposes without specific accuracy of their concentration.

(3) Temperature is expressed in $^{\circ}\text{C}$ (degree Celsius).

The temperature of *water bath* is 98-100 $^{\circ}\text{C}$, unless otherwise stated;

Hot water refers to that at a temperature of 70-80 $^{\circ}\text{C}$;

Slightly warm or warm water refers to that at a temperature of 40-50 $^{\circ}\text{C}$;

Room temperature is at a temperature of 10-30 $^{\circ}\text{C}$;

Cold water refers to that at a temperature of 2-10 $^{\circ}\text{C}$;

Ice bath refers to that the bath temperature is about 0 $^{\circ}\text{C}$;

Allow to cool refers to that the object is cooled to room temperature.

(4) The symbol "%" is used in the expression of percentage, usually by weight, but the percentage of solutions, unless otherwise stated, refers to the number of grams of solute in 100 ml of the solution. The percentage of ethanol refers to the percentage by volume at a temperature of 20 $^{\circ}\text{C}$. The following symbols may be used when needed;

% (g/g) expresses the number of grams of solute in 100 g of product/solution.

% (ml/ml) expresses the number of milliliters of solute in 100 ml of product/solution.

% (ml/g) expresses the number of milliliters of solute in 100 g of product/solution.

% (g/ml) expresses the number of grams of solute in 100ml of product/solution.

(5) The *Drop* of a liquid refers to the conversion that 1.0 ml of water is equivalent to 20 drops at the temperature of 20°C.

(6) The expression “(1→10)” following the solution refers to a solution of 10 ml produced by adding a sufficient quantity of solvent to dissolve 1.0 g or 1.0 ml of a solute. It is understood to be an aqueous solution if the solvent is not specified. In case of two or more solvents are used as a mixture, a hyphen is inserted between different solvents indicated by names, and the symbol “:” between numerals in parenthesis expresses the proportion of each solvent by volume (weight) in the mixture.

(7) Ethanol refers to that of 95% (ml/ml) in strength, unless otherwise stated.

17. The atomic weights adopted for calculating the molecular weights and the conversion factors are the values recommended by the International Table of Relative Atomic Weights.

Packaging, labeling, directions for use, storage and transportation

18. Immediate packaging materials and containers, including stoppers, shall meet the relevant standards promulgated by the NRA. They shall be not toxic or harmful, and shall be clean and sterile, and not interact physically or chemically with the products nor shall they affect the quality of the products. The tightness of sealing of immediate containers for injection shall be validated with an appropriate method.
19. Labels and directions for use of biological products shall comply with the related regulations described in the General Requirements for Packaging of Biologics in this Pharmacopoeia.
20. Unless otherwise stated, biological products shall be stored at 2-8°C and protected from light. Shipping by cold-chain in a fastest way shall be adopted so as to reduce the time for transportation. Freezing shall be avoided for liquid products during shipping in winter.
21. Of each batch of products qualified in control tests, samples shall be retained in duplicate for further control tests whenever necessary, unless otherwise stated.
22. For clinical use, the diluent used to reconstitute the freeze-dried products shall be approved by the NRA.

Abbreviations

ATCC	American Type Culture Collection
ALT	Alanine aminotransferase
BSA	Bovine serum albumin
CCID ₅₀	Median (or 50%) infective dose of cell culture
CCU	Colour change unit
CFU	Colony forming unit
CFT	Complement fixation test
CH ₅₀	Median (50%) hemolytic unit of complement
CPE	Cytopathic effect
CMCC	China Medical Culture Collection

CRS	Chemical reference substance
DNA	Deoxyribonucleic acid
ED ₅₀	Median (50%) effective dose
ELISA	Enzyme linked immunosorbent assay
EU	Endotoxin unit
GMP	Good Manufacturing Practice
GLP	Good Laboratory Practice
HA	Hemagglutination
HI	Hemagglutination inhibition
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
i. c.	Intracerebral (ly)
i. d.	Intradermal (ly)
ID ₅₀	Median (50%) infective dose
IFA	Indirect fluorescent assay
Ig	Immunoglobulin
i. n.	Intranasal (ly)
i. m.	Intramuscular (ly)
IOU	International opacity unit
i. p.	Intraperitoneal (ly)
i. v.	Intravenous (ly)
IU	International unit
kD	Kilo Dalton
K _p	Distribution coefficient
LAL	Limulus amoebocyte lysate
Lf	Flocculation unit of toxin or toxoid
Lf/mg N	Flocculation units per milligram of total nitrogen
Lf/mg PN	Flocculation units per milligram of protein nitrogen
Lf/mg Pr	Flocculation units per milligram of protein
LgX	Log ₁₀ X
L+	Limes tod
LD ₅₀	Median (or 50%) lethal dose
MEM	Minimum essential medium
MHU	Minimum hemolysis unit
MLD	Minimum lethal dose
MOI	Multiplicity of infection
MVD	Maximum valid dilution
NRA	National Regulatory Authority
NCL	National Control Laboratory
PB	Phosphate buffer
PBS	Phosphate buffer saline

PERT	Product enhanced reverse transcription
PFU	Plaque forming units
PCR	Polymerase chain reaction
pH	Hydrogen ion exponent
PHA	Passive hemagglutination
PKA	Prekallikrein activator
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPHA	Reverse passive hemagglutination
s. c.	Subcutaneous (ly)
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoreses
SPF	Specific pathogen-free
U	Unit
VS	Volumetric solution
WHO	World Health Organization

Glossary of Terms for Biological Substances

This document is prepared to harmonize the terms and interpretations in the requirements for biological products of 2005 edition. The references are made for the preparation of this document such as the WHO Glossary of Terms for Biological Substances, Chinese GMP and the WHO Biological Requirements.

Adventitious Agents

Contaminating agents, including bacteria, fungi, mycoplasmas, endogenous and exogenous viruses, present in the inoculum or the substrate and /or materials used in production of biological product.

Antigenicity

Capacity for a substance to react with appropriate antibodies in a suitable *in vitro* immunological assay, such as flocculation, immuno gel diffusion, ELISA.

Attenuated Strains

A bacterium or virus, the virulence of which has been suitably reduced or abolished for a given host.

Batch

A part of final lot derived from a final bulk although a certain homogeneity is expected to exist between all the final containers of the batch, the degree of homogeneity may be less than that of the final lot, especially since the risks of contamination during filling may differ between final lots.

Biologics (Biologicals)

Biological Products refer to the drugs prepared from biological substances such as microbes, cells, tissues or fluids from animal or human origin by using conventional methods or biotechnological techniques, for the purposes of prophylaxis, treatment and diagnosis of human diseases. Biological products include bacterial vaccines (including toxoids), viral vaccines, antitoxins and antisera, blood products, cytokines, growth factors, enzyme, *in vivo* and *in vitro* diagnostic reagents, as well as other biological products such as toxins, antigens, allergens, monoclonal antibodies, antigen-antibody complexes,

immunoregulators and probiotics, etc.

Biological References

See the Requirements for Preparation and Calibration of National Standard Substances of Biologics described in the General Requirements of this edition.

Biological Standards

See the Requirements for Preparation and Calibration of National Standard Substances of Biologics described in the General Requirements of this edition.

Bivalent Vaccine or Polyvalent Vaccine

A vaccine formulated with two or more different types (or groups) of antigens.

Blood Products

Plasma protein fractions obtained by separation and purification of healthy human plasma or plasma of healthy individuals immunized with specific vaccine, or made with recombinant DNA techniques. Blood cell components are also regarded as blood products. Both plasma protein fractions and blood cell components can be used for treatment and passive immunoprophylaxis.

Bulk

The homogeneous material which is used to prepare final formulation or final bulk. It is obtained from one or more single harvests, is generally purified and may yield one or more final bulks. If it contains microorganisms, it is usually referred to as bulk suspension. If the bulk has been concentrated, a dilution step precedes the final formulation. In the case of some multivalent products (e. g. trivalent polio) it is derived from the mixing of monovalent bulk components.

Carrier

A molecule, generally a protein, to which a microbial polysaccharide is chemically linked for the purpose of eliciting a T cell dependent immune response and thus modifying the humoral immune response to the polysaccharide.

Cell Bank

A system whereby successive batches of product are manufactured by culture in cells derived from a primary cell bank (PCB) and a master cell bank (MCB), which have been fully characterized for identity and absence of adventitious agents. A number of containers from the master cell bank are used to prepare a working cell bank. In a finite passage system the production is validated for a passage level or number of population doublings beyond that achieved during routine production.

Cell Line

Any population of cells—other than those of the primary culture—prepared either by the first subculture or at any stage during the serial subcultures of a primary culture. Such a population of cells is commonly heterogeneous.

Cell Strains

Cell populations that have a finite capacity to replicate, do not produce tumors when inoculated into

experimental animals, have the karyology of the tissue of origin and are anchorage dependent.

Combined Vaccines

A vaccine formulated with two or more different antigen bulks in proportion as an effective immunogen with multiple immunogenicity.

Continuous Cell Lines

Cell populations that appear to be capable of unlimited *in vitro* propagation, do not have the karyology of the tissue of origin and are usually anchorage independent.

Expiry Date

The date after which it is not longer possible to guarantee that the product meets each of the manufacturing and control requirements especially the requirement for potency.

Final Bulk

The finished homogeneous material prepared from one or more bulks present in a single container from which the final containers are filled.

Final Product

Biological product which has undergone all stages of manufacture, including packaging and quality control and has been released for use.

Good Manufacturing Practice

Good manufacturing practice (GMP) is that part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the NRA. GMP rules are directed primarily to diminishing the risks, inherent in any biological production that cannot be prevented completely through the testing of final products. Such risks are essentially of two types: cross-contamination and errors in labeling.

Homogeneity

The condition of being of uniform structure and composition with respect to one or more specified properties.

Immunogenicity

Capacity for a product upon administration to elicit an immune response. In the case of vaccines, such reactions cause the desired appearance of a specific humoral immunity (antibody production by B cells), or cellular immunity (various T cell proliferation) or both, and generally result in the protection of individuals against infectious diseases.

Master Cell Bank (MCB)

A quantity of fully characterized cells of human, animal or other origin appropriately stored, usually stored frozen at -130°C or below, in aliquots of uniform composition, derived from a primary cell bank and usually used for the production of a manufacturer's Working Cell Bank (MWCB). Any containers once removed from the cell bank shall not be put back, no matter if they have been opened.

Master Seed Lot

A quantity of virus or bacteria derived from, or used to prepare, an original vaccine; the virus or bacterial suspension has been processed as a single lot to ensure a uniform composition and is fully characterized. The master seed lot is used for the preparation of working seed lots.

Manufacturing

A complete cycle of production of a pharmaceutical and biological product.

Manufacturer

Holder of a manufacturing authorization.

National Regulatory Authority (NRA)

The drug regulatory department under the State Council responsible for drug supervision and administration nationwide.

National Control Laboratory (NCL)

The drug testing institute, directly under the State Council, responsible for drug testing required for conducting drug review and approval and controlling drug quality in accordance with law.

Original Vaccine

A vaccine prepared according to the manufacturer's specifications and shown in clinical trials to be safe and immunogenic.

Packaging Material

Any material used in the packaging of a biological product, including inner and outer packaging materials, label, marks against falsity and package inserts.

Plasma

The liquid part remaining after the separation of the cellular elements from one unit of blood collected in a closed receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulant blood in an apheresis procedure.

Plasmapheresis and Cytapheresis

A kind of technique for collection of the plasma or specific cell components (s) from the whole blood by a separating machine with simultaneous returning the uncollected component (s) into the donor.

Plasmid

An autonomously replicating, circular, extrachromosomal DNA element. It usually carries a number of genes, some of which may confer resistance to various antibiotics; such resistance is often used to discriminate between organisms that contain the plasmid and those that do not.

Primary Cell Bank (PCB)

A quantity of fully characterized cells of human, animal or other origin usually stored frozen at -130°C or below in aliquots of uniform composition derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank. Any containers once removed from the cell bank shall not

be put back, no matter if they have been opened.

Primary Seed Lot

A quantity of viral or bacterial suspension, which has been identified by its origin, history and biological characteristics and demonstrated to be safe and of good immunogenicity in clinical study or the viral/bacterial suspension used for the preparation of original vaccines, can be used to prepare master seed lot. The suspension shall be processed as one single batch with a uniform composition and fully characterized.

Potency

Expression of the predicted capacity of a product to achieve its intended role; it is based on the measurement of some attribute of the product and is determined by a suitable quantitative laboratory method. In general potencies of biological products tested by different laboratories can be compared in a meaningful way only if they are expressed in relation to an International standard or an appropriate Reference Material.

Raw Materials (Source Materials)

All the biological materials and chemicals (excluding subsidiary materials) used during production of biological products.

Recombinant DNA Products, rDNA Products

Products of rDNA technology are produced by genetic modification in which DNA coding for the required product is introduced, usually by means of a plasmid or a viral vector, into a suitable microorganism or cell line, in which that DNA is expressed and translated into protein. The desired product is then recovered by extraction and purification. The cell or microorganism before harbouring the vector is referred to as the host cell, and the stable association of the two used in the manufacturing process is referred to as the host-vector system.

Single Harvest

A quantity of viral or bacterial suspension derived from substrate (a group of animals, or a group of embryonated eggs or cell cultures) that was inoculated with the same viral or bacterial strains, incubated and harvested together from a single production run in successive sessions.

Sub-lot

One homogeneous final bulk is dispensed separately in several intermediate containers each of which is a sub-lot that shall then be dispensed in final containers. Or one homogeneous final bulk is dispensed directly in final containers through several filling machines simultaneously, and the sub-lots are defined based on the number of corresponding filling machines or freeze-drying chambers used.

Subsidiary Materials

All the subsidiary materials used during the formulation of biological products, such as adjuvant, stabilizer, excipient and so on.

Validity Period

The maximum period of the time permitted by the NRA for a released product to be available for clinical use; the expiry date appears on the label of the final container.

Vector

A piece of DNA that can direct its own replication within a host cell and to which other DNA molecules can be attached and thus amplified. Many vectors are bacterial plasmids; in certain instances a vector may be integrated into the host-cell chromosome following its introduction into the cell and is maintained in this form during the growth and multiplication of the host organism.

Whole Blood

Blood collected in an anticoagulant solution with or without the addition of nutrients such as glucose or adenine.

Working Cell bank (WCB)

A quantity of cells of uniform composition derived from the master cell bank at a finite passage level, dispensed in aliquot into individual containers and appropriately stored, usually frozen at -130°C or below, one or more of which can be used for production purposes. All containers are treated identically and, anyone removed from storage, shall not be returned to the stock.

Working Seed Lot

A quantity of virus or bacteria of uniform compositions derived from the master seed lot by a single passage by a method approved by the NRA. The working seed lot, which is composed of the dispensed suspension in aliquot, is used for production.

GENERAL REQUIREMENTS



Contents of General Requirements

Requirements for Bacterial and Viral Strains/Seeds Used for Production and	
Quality Control of Biologics	4
Requirements for Preparation and Calibration of National Standard Substances of Biologics	5
Requirements for Defining Batches of Biologics	7
Requirements for Filling and Lyophilization of Biologics	7
Requirements for Packaging of Biologics	9
Requirements for Storage and Shipping of Biologics	10
Requirements for Quarantine and Management of Horses Used for Production of Biologics	11
Requirements for Source Plasma of Blood Products	13
Requirements for Preparation and Control of Animal Cell Substrates Used for	
Production of Biologics	17

Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics

I General consideration

1. The bacterial and viral strains/seeds stated in this edition refer to the bacteria, viruses or rickettsiae, etc. which are used directly in the production and quality control of biologics. The bacterial and viral strains/seeds are classified according to Article 2 of the *Provisions for China Medical Culture Collection*.

2. Bacterial and viral seeds/strains and cells derived from human or animal, or strains and cells constructed by recombinant DNA techniques used directly in production and quality control shall be subject to the approval of the NRA. The bacterial and viral strains/seeds used in the production and quality control of biologics by manufacturer shall be preserved, tested and distributed by the NCL or by the institutions designated by the NRA. Where any change of strains/seeds is to be made, or any newly isolated or collected strains by the manufacturer are to be used by any manufacturer for the production and quality control of biologics, it shall be subject to the approval of the NRA upon the verification of the NCL. Any strains/seeds, which are intended to be used for new biologics, must go through the evaluation and approval by the NRA according to the *Provisions for Drug Registration* issued by the NRA.

3. The production of biologics shall be based on a seed lot system. The record, history, source and biological characteristics of primary seed lot shall be verified. The master seed lot shall be prepared by passage and propagation of primary seed lot, and working seed lot shall be prepared by passage and propagation of master seed lot. The biological characteristics of the working seed lot shall be in conformity with that of primary seed lot. Each lot of master seed and working seed shall be preserved, tested and used respectively according to the requirements described in individual monographs.

4. The laboratories responsible for the preservation and testing of the strains/seeds shall comply with the requirements for biosafety laboratory described in the *General Norm of Biosafety for Laboratories of Microbiology and Medical Biology* (WS 233-2002).

5. The quality assurance department of a manufacturer is responsible for the centralized management of the strains/seeds, and shall

submit a written report to the director of the manufacturer and send a copy to the NCL annually. The quality assurance department of a manufacturer shall carry out investigation periodically on the storage, testing and the use of strains/seeds, and if necessary, make spot-checks, or conduct inspections along with the production department.

II Registration of bacterial and viral strains/seeds

1. All the bacterial and viral strains/seeds shall be serially numbered individually by the NCL, and any arbitrary change or forging of the number shall be forbidden. Any seeds or strains without the above serial number shall not be used for the production or quality control of biologics.

2. There must be a strict registration procedure for custody of the strains/seeds, and a general account as well as ledgers shall be set up. The bacterial and viral strains/seeds, when received, shall be given a serial number promptly, and its scientific name, strain name, history, source, characteristics, lot number, usage, date of passaging and/or of lyophilization and the number of containers shall be recorded in detail. The passaging, lyophilization and distribution of the strains/seeds shall be clearly registered in time and traceable, and the respective number of various strains/seeds in stock shall be checked periodically during the preservation period.

3. Generally, the bacterial and viral strains/seeds, when received, shall be subject to control tests promptly. The bacterial strains/seeds preserved in the medium shall be tested immediately.

III Control tests on bacterial and viral strains/seeds

1. The bacterial and viral strains/seeds used for the production shall be tested periodically according to their respective requirements.

2. All the results of the control tests of strains/seeds shall be timely recorded in a form designed for the quality control of bacterial and viral strains/seeds.

3. The virulent and attenuated strains/seeds of the same or different genera shall not be manipulated in the same clean room at the same time. The bacterial and viral strains/seeds of Class 1 and Class 2, bacilli and fungi must be manipulated in a strictly isolated laboratory with appropriate biosafety level, and in the room for experimental animals. The protection approaches for the operators shall be strengthened. Manipulations of live bacteria and viruses shall follow strictly the *Regulations for Handling Live Bacteria and Live Viruses*.

4. Manipulations of the bacterial and viral strains/seeds of Class 3 and Class 4 shall be carried out in a dedicated laboratory with appropriate biosafety levels as stipulated in respective requirements.

IV Storage of bacterial and viral strains/seeds

1. The bacterial and viral strains/seeds, after control testing, shall be preserved with appropriate methods, preferably at a low temperature or by lyophilization based on their respective characters.

2. The strains/seeds, which can not be preserved by lyophilization, shall be stored in duplicate or in two different media for periodic and regular transplantation and passage, respectively. The containers with bacterial strains/seeds preserved in medium shall be sealed with paraffin or by fusing.

3. The passage and lyophilization of the preserved bacterial and viral strains/seeds shall be recorded in a specified form.

4. A label, which bears the serial number, number of passages, lot number and storing date of the strains/seeds must be stuck firmly on each container.

V Destruction of bacterial and viral strains/seeds

The seeds/strains which are not worth retaining can be destroyed. The destruction of primary seed lot, master seed lot and working seed lot which are made of microbes of Class 1 and 2 shall be subject to the approval by the director of institution and the consent of the health administrative authority of the state or the health department of the people's government of a province, autonomous region or municipalities under the State Council. The destruction of seeds/strains of Class 3 and 4 shall be subject to the approval by the director of institution. The records of any strains/seeds destroyed shall be cancelled, and the reason for and the date of destruction as well as the way used shall be recorded.

VI Exchange of bacterial and viral strains/seeds

1. The bacterial and viral strains/seeds shall be preferably lyophilized and sealed under vacuum before release. If impossible, the viral strains/seeds in the form of tissue or cell suspension, and the bacterial strains/seeds preserved in medium in a tightly sealed container can be released. The outer package shall be unbreakable.

2. All the bacterial and viral strains/seeds exchanged between manufacturers and other institutions, which are used directly for the production and quality control of biologics, shall be subject to the consent of the NCL upon examination.

VII Request for and distribution of bacterial and viral strains/seeds

1. Request for or shipping strains/seeds shall be implemented according to the *Provisions for China Medical Culture Collection*.

2. When the strains/seeds used for production and quality control are distributed, a detailed record of history and the results from various control tests shall be attached.

Requirements for Preparation and Calibration of National Standard Substances of Biologics

I Definition

The standard substances of biologics refer to the biological standards or references used for determination of potency, activity or content of biological products or used for identification and characterization of biologics.

II Classification of national standard substances

The national standard substances are divided into two classes.

1. *National Biological Standards* refer to the standard substances calibrated with the international standards or prepared domestically (if international standards are not available) which can be used for measuring the potency or toxicity of a given product. The biological activity is expressed in international units (IU) or in units (U).

2. *National Biological References* refer to biological diagnostic reagents, biomaterials or specific antisera calibrated with the international reference reagents or prepared domestically (if international reference reagents are not available) which can be used for the qualitative identification of microorganisms (or its derivatives) or for disease diagnosis. Biological references also refer to the reference materials used for the quantitative determination of biological potency of certain biological products, such as reference materials for titration of virus content of live measles vaccine, or of flocculation units of toxoid, by which the potency can be expressed in units (U) of specific activity rather than in international units.

III Preparation and calibration of standard substances

1. The laboratories and clean rooms for preparing standard substances shall comply with the requirements of the Chinese Good Manufacturing Practice for Pharmaceutical Products (GMP) or the Chinese Good Laboratory Practice (GLP).

2. The NCL is responsible for preparing and calibrating the standard substances, and applying to WHO for the international standards and international references, which shall be kept, used and distributed by the NCL.

3. Research and development of new standard substances

(1) Selection of source materials

The nature of the source materials of biological standard substances shall be identical to that of the sample to be tested. The source materials shall not contain any interfering matters. They shall be sufficient in quantity and of adequate stability and high specificity.

(2) Formulation, filling, lyophilization and sealing of containers

The formulation and dilution of the standard substances shall be performed as required. The stabilizers or other materials, which need to be added, shall be of no influence on the activity and stability of the standard substances as well as on the assaying process, and shall not volatilize during lyophilization.

The substances qualified in control tests shall be dispensed accurately with a precision of $\pm 1\%$.

The substances that need to be dried for preservation shall be sealed immediately after lyophilization. The residual moisture in the freeze-dried substances shall be not more than 3%.

It is necessary to ensure the consistency of potency and stability in each container during the course of filling, lyophilization and sealing.

(3) Calibration

① Collaborative calibration

The development and calibration of the standard substances to be established shall be conducted collaboratively in three experienced laboratories at least. The same protocols, methods and record forms shall be adopted unanimously by the participants, and the analysis of the calibrated results shall be performed statistically (the calibrated result necessitates five independent valid results at least).

② Confirmation of activity (potency unit or toxicity unit)

The activity is expressed usually by the mean value of the calibrated results obtained by participating laboratories. All the data shall be collected and analyzed statistically by the NCL and submitted to the NRA for approval.

(4) Study on stability

The accelerated destruction test shall be carried out to study the stability. The substances with different properties concerned shall be placed at different temperatures (4°C, 25°C, 37°C and -20°C) to test the activity at different period of storage. The activity of established standard substances shall be compared with that of the international standard substances periodically to find out if the activity decreases.

4. Preparation and calibration of the substitute lot of standard substances

(1) The NCL is responsible for the preparation and calibration.

(2) The biological and biochemical properties of the source materials for preparing the substitute lot of standard substance shall be identical as much as possible to those of the substituted lot.

IV Approval of the standard substances

1. The collaboratively calibrated results of a newly established standard substance shall be reviewed by the NCL and, if accepted, submitted to the NRA for approval and issuing the certificate of the standard substances.

2. The substitute lot of standard substance shall be reviewed and accepted by the NCL.

3. The newly established standard substance shall be released for use only after the certificate is issued.

V Labels and package inserts

1. Labels and package inserts shall be issued for the qualified standard substances by the quality assurance department of the NCL.

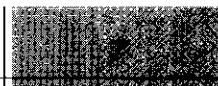
2. The name, lot number and the stated amount (the quantity of solid substance containing 1 IU, IU per mg, or IU per container) shall be indicated on the label.

3. Package inserts shall be attached to the standard substances and reference materials, and the following items shall be indicated in the package inserts: The name in Chinese and English, component and properties, filling quantity and the stated amount, lot number, storage conditions, usage, expiry date, and distributor, etc.

VI Request for and storage of standard substances

1. The request for the standard substances should be made directly to the NCL. The national standard substances are provided to the manufacturers for calibrating working standards or for quality control.

2. The standard substances shall be stored at a suitable temperature and humidity that shall be checked periodically and recorded.



3. The person specially assigned shall be responsible for keeping and releasing the standard substances.

Requirements for Defining Batches of Biologics

The batch number serves as a marker for defining and identification of batches of biological products. In order to avoid confusion or error, all final products shall be serially numbered in batch according to the Requirements, unless otherwise stated.

1. The batch number of biologics shall be compiled by the production department and approved by the quality assurance department.

2. The principle for coding batch number is: Year+Month+serial number. The 'Year' shall be four-figure number of the Gregorian calendar year, two-figure number for the 'Month'. Two or three digits can be used as the serial number according to the number of product lots produced by the manufacturer. Prior to the serial number a Chinese character or an English letter can be added to indicate some specified implications.

3. The constituents of a given batch of biologics must be completely identical, i.e. the origin and the quality of the biologics in any container of the same batch must be identical to that in any one among the rest containers. Therefore, the assessment of a whole batch of biologics can be made by sampling and checking part of the containers (except for single-donor and small-pool blood products).

4. Defining batch number

- (1) Before filling, the final bulk made at the last step of bulk mixing, formulation, and dilution or filtration after dilution of the product shall be serially numbered. If a given batch has to be divided into several big bottles, a sub-lot number suffixed to the given batch number, shall be given to each of bottles. The same product diluted, mixed, adsorbed and/or filtrated not at the same time shall not be defined as the same batch.

- (2) If the mixed or diluted product is filtered with more than two filters, the batch number (or sub-lot number) shall be defined by different filters. If the same product is filtered at different times, different batch numbers (or sub-lot numbers) shall be defined accordingly.

- (3) The product diluted in a tank shall be defined as one batch for filling with several filling machines. And the sub-lot numbers shall be defined respectively according to the different filling machines used.

- (4) For the same batch of product lyophilized with different freeze-dryers or at different times, the

sub-lot numbers shall be defined accordingly.

- (5) Another sub-lot number shall be defined to the product when any part of the filling set is changed during filling process.

5. The used apparatus for filling a given batch shall not be used for another batch before being washed and sterilized.

6. The batch number of the same product must not be repeated; and the same batch number shall not be used for the same product with different specifications.

Requirements for Filling and Lyophilization of Biologics

The Requirements are applied to all the injections of biologics which make up a majority in the forms of biological products. The filling requirements and the actual filling quantity of other forms, such as capsules, tablets, powder, eye drops, suppositories or those not mentioned in the requirements shall comply with the relevant requirements in the General Requirements for Preparations in the Appendix I of this Pharmacopoeia.

I Acceptance by quality assurance department

The final bulk to be filled and/or lyophilized shall be subject to the examination or testing by the quality assurance department. To those qualified in control tests a Notice for Filling shall be issued before filling or lyophilization after filling, unless otherwise stated specially.

II Containers and apparatus for filling and lyophilization

1. The quality of source materials of final containers for filling and lyophilization shall comply with the relevant national standards of packaging materials and containers for medicaments. The glassware shall be sterilized by autoclave at 121°C for 1 hour, or by dry heat at 180°C for 2 hours at least, or by other means with the same effectiveness. During sterilization there shall be no glass flakes falling off or alkaline substances separated out.

2. The filling apparatus and containers contacting directly with different products shall be washed separately. The filling apparatus as well as containers shall be used dedicatedly for sera, blood products, BCG or tuberculin, etc.

III Workshops for filling and lyophilization

1. The workshops for filling and lyophilization

shall comply with the requirements described in the *Chinese GMP for Pharmaceutical Products*.

2. The inactivated vaccines, recombinant vaccines, toxoids, and cellular extracts, when their inactivation or purification is completed, can be dispensed or lyophilized in the same workshop of filling or the same facilities for filling and lyophilization, but not at the same time as they are done for other products without living organism, provided that thorough cleaning and sterilization are conducted upon the completion of filling of each kind of product, and the validation of cleaning effectiveness shall be carried out periodically.

IV Personnel

Physical examination shall be carried out at least once a year for the personnel taking part directly in filling and lyophilization work. Those with active tuberculosis, infection of viral hepatitis or other infectious diseases, which could contaminate the product potentially, shall be forbidden to go in for filling and lyophilization work.

V Requirements for the product to be filled

1. The time elapsed from the last sterility test of the product to be filled shall not exceed 6 months. If it has elapsed over 6 months, the sterility test shall be repeated.

2. The labels of the products to be dispensed must be intact and distinctive. The product name and batch number must accord completely with those in the Notice for Filling. The mouth of container shall be wrapped tightly. The stopper shall be intact and no cracks shall be found on the container. The appearance of the product shall meet the requirements for the said product in monograph.

3. Meticulous measures should be taken to avoid contamination during storage and transfer of the products to be dispensed.

VI Requirements for filling

1. Prior to filling it is necessary to check everything concerned in order to avoid wrong or confused batches. Different products with the same colour or the same filling specifications, or live vaccines and other products shall not be filled simultaneously in the same room.

2. Aseptic manipulations shall be practiced strictly throughout the whole filling process. The product shall be dispensed directly as far as possible from the original container, unless otherwise stated. The filling of the product from one container should be completed preferably within the same day, if not, it shall be completed on the next day. The filling apparatus shall not be used consecutively for different sub-lots of the same product.

3. The filled containers, if ampoules used, shall be sealed by fusing immediately after filling; if glass or plastic vials are used, the container shall be stoppered and then sealed with sterilized aluminum cap immediately. The sealed containers shall be subject to inspection of leakage by reduced pressure or by other methods, unless otherwise stated.

4. During filling process, the ambient temperature for live vaccines and other temperature-sensitive products shall be below 25°C, or effective measures shall be taken to lower the temperature for the filled products, unless otherwise stated. The filled products shall be transferred to the cold room of 2-8°C as quick as possible (for those specified, the requirements in the relevant monograph shall apply).

5. The product containing adsorbent or other suspensions shall be maintained homogeneously during filling process.

6. The final containers and stoppers used in filling shall have no adverse influence on the potency, clarity and pH value of the said product.

7. Filling quantity

The actual filling quantity in the container shall be more than the stated value. It is necessary to supplement the filling amount of 100 ml with 4.0 ml, of 50 ml with 1.0 ml, of 20 ml with 0.60 ml, of 10 ml with 0.50 ml, of 5 ml with 0.30 ml, of 2 ml with 0.15 ml, of 1.0 ml with 0.10 ml and of 0.5 ml with 0.10 ml. It shall be guaranteed that the aspirated amount withdrawn from each container shall be not less than the stated value. For antitoxin, in addition to the above mentioned supplements, 10% or 20% shall be supplemented in terms of units.

The actual quantity of product in prefilled syringe shall be not lower than the stated value.

VII Requirements for lyophilization

1. The lyophilization process and the lyophilization curve adapted to the characteristics of the product shall be selected, and the freeze-dryer should be equipped with an automatic scanning device for recording. It is required that strictly aseptic operation be conducted during the whole process of lyophilization of any product.

2. The appropriate excipient shall be selected according the property of different products. The excipients to be used shall comply with the requirements of the current Chinese Pharmacopoeia or relevant national standards. The kind and the amount of excipient to be used in the product shall be innocuous, and shall have no adverse influence on the safety and efficacy of the product or shall not interfere with the stated testing methods.

3. Containers sealed under vacuum shall be subject to the test of vacuum, and those nitrogen-

filled shall be adequately filled with nitrogen. The purity of nitrogen shall be not less than 99.99%.

VII Cards and records for filling and lyophilization

The filling and lyophilization cards shall be filled in for each batch of filled product with the product name, batch number, sub-lot number, specifications, filling date, etc. At the same time the filling and lyophilization records shall also be kept, including the signatures of staff responsible for filling, sealing, stoppering and capping as well as the signatures of those responsible for checking.

IX Sampling for sterility test

Each batch or each sub-lot of filled and freeze-dried product shall be subject to sterility test (Appendix III A). Samples shall be taken randomly at the early, middle and late stages during filling process, or from separate compartments of the same freeze-dryer for sterility test which will be performed by the quality control department. The test samples of different freeze-dried products in different compartments of freeze-dryer shall be taken separately for sterility and moisture content tests.

Requirements for Packaging of Biologics

I General Requirements

1. Packaging of biological products shall be performed according to the related regulations described in the *Provisions for the Management of Package Inserts and Labels of Drugs* issued by the NRA.
2. The facilities for packaging shall comply with the requirements of the current *Chinese GMP for Pharmaceutical Products*. Packaging materials shall meet the criteria for drug packaging materials and containers set forth in the official regulations.
3. The filled or lyophilized products can be packaged only when the Notice for Packaging is received based on that the products are qualified in control tests and the comprehensive evaluations are made by the quality assurance department, unless otherwise stated.
4. There shall be segregations in the packaging site where several packaging lines are running simultaneously. The products with similar appearance shall not be packed on the neighboring lines. There shall be a label on each packaging line

indicating evidently the name and batch number of the product being packed.

II Visual inspection

1. The sealed containers shall be subject to inspection of leakage by reduced pressure or other methods. The containers shall not be sunk in a liquid while reduced pressure method is used.
2. The products shall be subject to the visual inspection before packaging according to the respective requirements described in the monograph. The requirements and standards for the inspection of products are as follows.
 - (1) Fluorescence lamp (of 1000-3000 LX) is used for the inspection of containers, and the adjustment of background and intensity of illumination should depend on the character of the product.
 - (2) The workers responsible for the inspection of containers shall be subject to vision test once half a year and the visual acuity shall be equal to or above 4.9 and the corrected visual acuity shall be equal to or above 5.0, and they shall be of no colour blindness.
 - (3) Any product with abnormal colour, transparency or concentration, or with foreign matters, clumps not dispersed on shaking, crystals separated out, sealing defect, burned black tip of the container in sealing or cracks in the containers shall be discarded, for those specified, the requirements in the relevant monograph shall apply.

III Label

1. The labels of drugs shall comply with the requirements set forth in the *Drug Law of the People's Republic of China* and the certain regulations issued by the NRA. The content of label for different package shall be in conformity to the above mentioned regulations.
2. The description of the drug label shall be compiled by the manufacturer based on the drug package inserts approved by the NRA. The content shall not include those beyond the approved content of package inserts, no unrelated wording or pattern shall be added.
3. The content and pattern of drug labels on both inner and outer packages shall comply with the relevant regulations issued by the NRA.

IV Packaging

1. Before packaging, the labels of containers or the stamp with expiry date shall be prepared according to the Notice for Packaging issued by the quality assurance department. The words printed on the labels must be clear.
2. To prevent wrong package, it is necessary to check the batch number of the product against the

Notice for Packaging. In the process of packaging, any product with abnormal appearance, or foreign matters, or the containers with leakage shall be discarded.

3. The ambient temperature for packaging shall be at 25°C or below, for those specified, the requirements in the relevant monograph shall apply.

4. The labels shall be stuck firmly on the containers not easy to fall off and not to be blurred. The content of label shall not be altered or supplemented by means of sticking or clipping and pasting. The printed words, numbers and letters directly on the containers shall be clear and discernable.

5. For different products or the same product with different specifications, their package inserts and labels shall be different in colour or in form in order to be discernable.

6. The content of label of the outer package shall be printed directly on the carton. The batch number and expiry date shall be printed directly on the carton with appropriate numbering machine, and the printed words shall be clear and not easy to be blurred.

7. The clearance of the packaging site on completion of an operation shall be carried out and the clearance record shall be filled in.

8. The packed products shall be stored in a control area before the certificate for Qualification is issued, and the packed product qualified in control test shall be removed to the warehouse for final product and the form for stock is filled in.

9. A package insert shall be attached in each smallest pack.

V Package inserts

1. Package inserts of drugs shall comply with the requirements set forth in the *Drug Law of the People's Republic of China* and the certain regulations issued by the NRA. The package inserts shall be compiled according to the approved instructions by the NRA.

2. The content of package inserts of the products for prophylaxis shall include the drug name (including adopted Chinese name, English name and Chinese phonetic alphabet), constituents and characters, eligible, function and usage, specifications, administration and dosage, adverse reactions, contraindications, precautions, storage, package, validity period, standard for implementation, product license number and manufacturer (name, address, zip code, telephone number, fax, web site).

3. The content of package inserts of the products for therapeutic use shall include the drug name (including adopted Chinese name, English name and Chinese phonetic alphabet), constituents and

characters, indications, specifications, administration and dosage, adverse reactions, contraindications, precautions, storage, package, validity period, standards for implementation, product license number and manufacturer (name, address, zip code, telephone number, fax, web site).

The content of package inserts shall also include the drug administration for women during lactation period or in pregnancy, drug interactions, the drug for pediatric use and for geriatric use, overdosage, study on pharmacology, toxicology and pharmacokinetics. The above mentioned contents shall be compiled according to the regulations issued by the NRA.

4. The content of package inserts of diagnostic reagents *in vivo* shall include the drug name (including adopted Chinese name, English name and Chinese phonetic alphabet), constituents and characters, function and usage, eligible, administration and dosage, result evaluation, contraindications, adverse reactions, precautions, specifications, storage, package, validity period, product license number and manufacturer (name, address, zip code, telephone number, fax, web site).

Notice: **Specifications** refer to the potency (or content and potency) of the active ingredients and the filling quantity (or volume of diluent for reconstitution of the freeze-dried product) in each container.

Requirements for Storage and Shipping of Biologics

The Requirements shall be followed for biological products during production, staging period for testing and for sale, and during distribution so as to keep the stability of product quality.

1. Any manufacturer shall, according to the *Chinese GMP for Pharmaceutical Products*, have dedicated refrigerating facilities for storage of harvests, bulks, final bulks and final products of biologics.

2. The harvests, bulks, final bulks and final products listed below shall be separately stored. There shall be facilities with segregations in order to avoid confusion.

(1) The harvest or bulk that has not been processed or is being processed shall be stored separately by the production department.

(2) The ready made bulk or final bulk shall also be stored separately by the production department before control test results are concluded.

(3) The final bulk to be filled, the filled final bulk to be tested or those qualified in control tests



but to be packed shall be stored separately by filling and packaging departments.

(4) The packed products qualified in control tests shall be stored in the warehouse for final products.

3. The containers with a given harvest, bulk, final bulk or final product shall be labelled indicating evidently the product name, batch (sub-lot) number, specifications, quantity as well as the date of storage.

4. Persons specially assigned shall be in charge of the stocks of bulks, final bulks and final products. The storage site card and inventory record shall be established for each kind of stored bulks, final bulks and final products. The person in charge shall fill in record and sign timely upon acceptance and release of stocks.

5. The mouth of the container containing bulk or final bulk shall be wrapped tightly or sealed.

6. Bulks, final bulks or final products shall be stored under the specified conditions (temperature, humidity and light protection) according to the requirements of the said products. Temperature and humidity of the stock rooms shall be recorded as scheduled. Generally the storage temperature shall be 2-8°C, unless otherwise stated.

7. Persons specially assigned shall be responsible for the management of warehouses for bulks, final bulks and final products.

8. The bulks, final bulks or final products to be tested must be marked evidently with "RESERVE for TESTING".

9. The bulks, final bulks or final products unqualified in control tests shall be marked evidently with "UNQUALIFIED" and shall be disposed in time according to the relevant stipulations.

10. The bulks, final bulks or the final products qualified in control tests shall be marked evidently with "QUALIFIED" and shall be dealt with according to the relevant stipulations.

11. Shipping of biologics shall follow the principles below.

(1) The quickest way shall be adopted for shipping so as to shorten shipping period.

(2) Shipping shall be under refrigeration.

(3) Freezing shall be prevented during shipping biologics in winter season.

Requirements for Quarantine and Management of Horses Used for Production of Biologics

The Requirements apply to the quarantine, immunization, raising and management of horses and mules used for the preparation of antitoxins and

antisera.

The areas for the quarantine and immunization of horses and mules shall be separated strictly. The equipment and apparatus used in different areas shall be dedicated. Visitors and personnel not involved in or other kinds of animals are not allowed to enter these areas. The veterinarians trained professionally shall be responsible for the quarantine, raising and management, treatment and autopsy of the animals as well as the technical work concerned.

I Purchase and shipment

1. Purchase of animals

(1) The animals, i. e. horses and mules without any infectious diseases, shall be healthy and good in nutrition, and at the age of 4-15 years. The animals in black, white or pale colour are not suitable for the production of prophylactics and therapeutics.

(2) Animals shall not be purchased from the epizootic areas.

(3) The animals shall be tested on spot for glanders infection with mallein applied to the conjunctiva by eyedrops. The tests for equine infectious anemia and infections caused by *Salmonella abortus equi* shall be conducted, if permits.

(4) The animals that have received penicillin or human blood products shall not be purchased.

2. The selected animals shall be isolated and immunized with tetanus toxoid.

3. Personnel specially assigned are responsible for the shipment of animals. The vehicles for shipment shall be disinfected in advance. During shipment much attention shall be paid to safety.

II Quarantine

1. Quarantine of new animals

(1) The new animals entering the quarantine area shall be numbered, branded, quarantined, trained and inoculated with necessary vaccines for prophylaxis.

(2) Animals shall be quarantined for 90 days. During this period, besides systemic physical examination, the animals shall be subject to the following tests according to the methods and standards described in relevant regulations issued by the Ministry of Agriculture of People's Republic of China.

① Glanders

Eyedrop test with mallein shall be carried out. If necessary, other tests such as anaphylactic test or complement fixation test shall be performed.

② Equine infectious anemia

Complement fixation test or agar gel diffusion test shall be carried out, and fluorescent antibody test may be used.

③ Brucellosis

Agglutination test shall be carried out.

④ Salmonellosis

Agglutination test shall be carried out.

Routine tests and tests for other infectious diseases shall be carried out, if necessary.

2. The immunized animals shall be tested for glanders once or twice a year, for equine infectious anemia at least twice a year (prior and post to the fly and mosquito season respectively). Testing for malignancy and other infectious diseases shall be performed, if necessary.

3. The animals with positive or suspicious results in any tests shall be disposed promptly with effective measures and shall not be used for production.

III Immunization and blood collection

1. Horses used for immunization

(1) The horses used for immunization shall comply with the requirements given in Sections I and II of the Requirements.

(2) Immunization or blood collection shall be suspended immediately whenever the horses are found with infectious diseases or other serious diseases.

(3) The horses which failed to response to one kind of antigen after immunization may be immunized with other kinds of antigens or shall not be used for production.

(4) Horses used for the production of antitoxins and antisera must not be treated with penicillin or streptomycin.

2. Antigen and adjuvant

(1) The antigens with satisfactory antigenicity shall be used for immunization such as bacteria, viruses and refined toxoids or toxins. If necessary, antigens undetoxified or detoxified incompletely, or polymer of antigens may be used for immunization.

(2) Different kinds of antigens shall be evidently marked for strict distinguishment.

(3) Antigens shall be stored at 2-8°C in dark place; filling and formulation shall be carried out aseptically. If contaminating organisms are found, the antigens shall be discarded.

(4) The containers or syringes which have been used for the antigens undetoxified or detoxified incompletely must be sterilized before cleaning.

(5) The adjuvant for immunization shall be of good quality, safety, with high efficacy, without antigenicity, and shall contain no macromolecular components derived from human origin.

3. Immunization and blood collection

Operations shall be conducted following strict checking procedures. The horse blood shall be collected in such a way as to minimize the risk of contamination.

(1) Primary immunization

The primary immunization for horses shall be performed before hyperimmunization. An implementation plan shall be drafted according to different antigens to be used and to the practical conditions as well as the experiences.

(2) Hyperimmunization

The schedules for effective hyperimmunization and blood collection shall be laid down on the basis of the practical conditions of primary immunization and previous immunization protocols.

(3) Blood collection

Blood shall be collected from successfully immunized horses and the potency of the serum titer shall be not less than the standards described in relevant monograph. The quantity of blood to be collected shall depend on the health condition and body weight of the horse. Generally, 14-20 ml per kg body weight can be collected. An appropriate anticoagulant shall be added into the collected blood.

The horses used for blood collection should not be fed with fine forage at least 6 hours prior to the collection. The horses suffering from serious jaundice or other serious diseases shall not be used for blood collection.

IV Plasma separation

1. Plasma separation shall be carried out under aseptic conditions; it is preferably to separate the plasma from each horse individually. The plasma shall be sampled for sterility test and determination of titer. An appropriate preservative shall be added and the plasma shall be stored at low temperature in a dark place.

2. Care shall be taken to prevent blood cells mixing into plasma during blood separation. Plasmas with serious hemolysis or serious jaundice shall not be pooled.

3. The horses which are found suffering from infectious anemia shall be investigated and traced. The plasma collected after the last quarantine as well as the final bulks and final products contaminated with the plasma shall be discarded. The horses which are found suffering from carcinomas or glanders shall be investigated and traced. Plasmas collected within 3 months before finding of these kinds of diseases as well as the final bulks and final products contaminated with the plasma shall be discarded.

4. The containers, apparatus and solutions which contact directly with horse blood and plasma shall be sterile. Care shall be taken to prevent contamination by pyrogen or toxic substances.

5. The chemicals added to horse blood or plasma shall comply with the relevant specifications, stipulations described in the current *China Pharmacopoeia* and other relevant national standards.

6. Potency determination

The titer of horse immune serum or plasma can be determined with an appropriate method. The results shall be in compliance with those determined with the methods stated in the relevant Appendix of this edition for respective antitoxins or antisera.

V Management for horses

1. The immunized animals shall be fed with protein and vitamins enriched forage. The digestible protein in daily forage for each animal shall be not less than 720 g (contained in about 4 kg of fined forage). The animals should take appropriate physical exercise. The stall, stadium, water trough, feeding trough and the animals shall be kept clean. The stall and stadium shall be disinfected periodically. And the animals shall be weighed and have the hoofs cut periodically. The workers shall take good care of the animals and pay attention to their health status.

2. Attention shall be paid to the safety in the area where animals are raised. The forage grass shall be kept properly and prevented from rottenness, and fires shall be avoided at the forage stock site.

3. The blood can be collected from the animals that suffer from non-infectious diseases, or the immunization can be given provided that the veterinarian consents.

4. The following principles shall apply to dealing with the animals that suffer from infectious diseases. Measures for prevention and treatment in detail shall be taken.

(1) The sick animals or the animals with positive results in any tests found in the early stage shall be disposed promptly. It is necessary to isolate those suspected animals for observation, and effective measures for prevention shall be adopted for the animals with negative results.

(2) The management and nutrition for the animals shall be strengthened.

(3) The corpses of animals with fulminating infectious diseases or with positive results in the tests shall be incinerated or buried deeply, and their feces and the environment shall be strictly disinfected. The source of the disease shall be searched so as to refrain from the disease prevalence. A report shall be submitted to the superior. The event shall be handled according to the regulations issued by the Ministry of Agriculture in cooperation with local veterinary institutions.

5. The blood can be totally collected if the horses show severe reactions after immunization, weak constitution intolerable for continuing immunizations, and those suffer from incurable, non-infectious diseases, rupture of liver; and those in other special conditions.

6. Autopsy

The animals that die in collection of total blood or illness (infectious diseases excluded) shall be

handled in a specified laboratory and disposed after autopsy by veterinarian, and if necessary, pathological examination shall be conducted.

The blood or plasma shall be discarded if there is evidence of infectious disease or malignant tumor in autopsy of the animals.

The animal corpses shall be disposed according to the regulations in the *Tentative Requirements for Hygienic Examination of Meat Products* issued jointly by the Ministry of Agriculture and the Ministry of Health of the People's Republic of China.

Requirements for Source Plasma of Blood Products

Source plasma for preparing plasma protein preparations refers to the plasma collected from healthy human by means of plasmapheresis.

I Plasma donors

In selecting individuals for plasma donation it is important to determine whether the person is suitable to donate blood by means of inquiring his health condition, physical examination and laboratory findings. Only the experienced or well-trained physician shall decide the eligible donors based on the above information. The candidate for donation shall be informed about the methods and process of collecting plasma, the likelihood of reactions and potential risks during plasma collection. The form of physical examination can only be given to the candidate provided that he or she has consented orally or in written form to donation of plasma, and then the physical examination can be undertaken. Individuals who pass the physical examination and laboratory testing can be accepted as donors of plasma.

(I) Inquiry about health status

1. Individuals with the following conditions and history shall be excluded from donation:

(1) Weak constitution, frequent dizziness, dim-sighted, tinnitus, faint during puncture and bleeding, or frequent fainting and with a history of Meniere's syndrome.

(2) Venereal diseases, leprosy, AIDS or anti-HIV-1 and/or anti-HIV-2 positive.

(3) A history of hepatopathy, HBsAg positive, anti-HCV antibody positive, or ALT elevation in two consecutive determinations (a recovered patient with hepatitis A for over one year whose determinations of ALT are normal for three consecutive times at intervals of one month can be a candidate for plasma donation).

(4) Recurrent allergic diseases, urticaria, bronchial

asthma, or drug allergy (the patient with simple urticaria not at acute stage can be a candidate for plasma donation).

(5) A history of pulmonary tuberculosis, renal tuberculosis, tuberculosis of lymph nodes, or tuberculosis of bones.

(6) A history of cardiovascular diseases, various heart diseases, hypertension, hypotension, myocarditis, or thrombophlebitis.

(7) Respiratory system diseases such as chronic bronchitis, emphysema, bronchiectasis, or functional insufficiency of lung.

(8) Gastric ulcer and duodenal ulcer, chronic gastroenteritis, acute and chronic nephritis, chronic urinary system infections, nephrotic syndrome, or chronic pancreatitis.

(9) Various blood diseases such as anemia, leukemia, polycythemia vera or various hemorrhagic and blood coagulating diseases.

(10) Endocrinopathy or metabolism disorders such as hyperthyroidism, acromegaly, or diabetes insipidus, diabetes mellitus, etc.

(11) Organic diseases in nervous system or psychosis such as CJD, vCJD, encephalitis, sequelae of brain trauma, epilepsy, schizophrenia, hysteria, or severe neurasthenia, etc.

(12) Parasitosis or endemic diseases such as kala-azar, schistosomiasis, filariasis, hookworm disease, taeniasis, paragonimiasis, Ke-shan disease, Kaschin-Beck disease, etc.

(13) Malignant tumor, or benign tumor affecting health.

(14) A history of nephrectomy, splenectomy, pneumonectomy, cholecystectomy or gastrectomy, etc.

(15) Contacting with harmful substances or radioactive substances.

(16) Drug abuse, homosexuality or multiple sexual partners.

(17) A history of treatment with hormones derived from animal hypophysis such as growth hormones, gonadotropic hormones, thyroid-stimulating hormones; or transplantation of organs including cornea, bone marrow or dura mater etc.

(18) Other diseases considered by the physician.

2. Postponement of donation for those with one of the following conditions:

(1) Donors with a history of tooth extraction or other minor surgery within half a month prior to donation;

(2) Women within 3 days before or after menstruation, with irregular menses, during pregnancy, in less than 6 months after abortion, or in less than one year from parturition or lactation;

(3) Individuals in less than one week after recovery from common cold or acute gastroenteritis, in less than one month after recovery from acute urinary system infections, or in less than half a year from pneumonia;

(4) Individuals from endemic and high risk areas

of certain infectious diseases or the areas specified by the anti-epidemic sectors; individuals in less than half a year after recovery from dysentery; individuals in less than one year after recovery from typhoid fever or brucellosis; or individuals with a history of malaria within 3 years.

(5) Individuals who have received blood transfusion therapy shall be excluded from donation within 2 years.

3. Collection of plasma from the donor after immunization

The plasma from symptom-free donors who have been immunized recently can be accepted with the following exceptions:

(1) Those receiving live attenuated vaccines such as measles, mumps, yellow fever, poliomyelitis or hepatitis A vaccine shall be excluded until 2 weeks after the last immunization or injection. Those receiving live attenuated rubella vaccine and rabies vaccine shall be excluded until 4 weeks after the last injection. Those who had been bitten by rabid animals receiving rabies vaccine for post-exposure treatment shall be excluded until one year after the last injection.

(2) Those receiving passive immunization using animal serum products shall be excluded until 4 weeks after the last injection.

(3) Donation need not be postponed for the healthy individuals who has produced antibody after hepatitis B immunizations.

(II) Physical examination

1. Age

18-55 years old.

2. Body weight

Male: Not less than 50 kg; female: not less than 45 kg.

3. Blood pressure

Blood pressure: 12-20 kPa/8-12 kPa (90-140 mmHg/60-90 mmHg);

Pulse pressure: above 4 kPa (30 mmHg).

4. Pulse

60-100 beats/min.

5. Body temperature

Normal.

6. Thorax

Normal heart (including physiological heart murmur), normal in fluoroscopy of lungs (calcification of lung tuberculosis for more than 3 years). Fluoroscopic examination shall be carried out once a year. Fluoroscopy for chest is required for new donors.

7. Abdomen

Normal, no splenohepatomegaly, no mass and no tenderness.

8. Others

Normal development, no jaundice, no infectious skin diseases, no enlargement of superficial lymph nodes, no cervical lymph node enlargement and no



Kaposi sarcoma in limbs.

No serious illness in eyes, ears, nose and oral cavity, no thyroid enlargement and no serious deformity in limbs, no redness and swelling and functional disorder in joints.

(III) Standards of laboratory findings

1. Hemoglobin

Copper sulfate method shall be used.

Male; not less than 125 g/L; female; not less than 115 g/L.

2. Serum protein

The serum protein shall be not less than 60 g/L determined with Biuret method (Appendix VI B, method 3).

3. Blood grouping

The approved anti-A and anti-B grouping reagents shall be used. The blood grouping for ABO shall be determined by testing the red cells with anti-A and anti-B serum (forward method) and by testing the serum or plasma with known group A red cells and known group B red cells (reverse method).

4. Alanine aminotransferase (ALT)

It shall be not more than 25 units with Reitman-Frankel method or other approved methods.

5. Hepatitis B surface antigen

The result shall be negative detected with the approved diagnostic kit.

6. Serum electrophoresis

It shall be performed once a year, and the electrophoretic profile shall be normal with the albumin content of not less than 50%.

7. Syphilis

The result shall be negative detected with the approved diagnostic kit.

8. Anti-HIV-1/anti-HIV-2

The result shall be negative detected with the approved diagnostic kit.

9. Anti-HCV

The result shall be negative detected with the approved diagnostic kit.

II. Plasma collection

1. Plasmapheresis center

The plasmapheresis center shall be established in conformity with the requirements of the Minimal Standards for Plasmapheresis Center issued by the Ministry of Health of the People's Republic of China.

2. Materials for plasma collection

(1) All the disposable sets that contact directly with blood or plasma shall be sterilized and pyrogen-free. Each lot of sets shall be sampled for bacterial endotoxin test and the test result shall meet the requirements (100 ml of sodium chloride solution for injection shall be passed through the

set, the bacterial endotoxin content in the effluent shall be less than 0.5 EU/ml determined by the limit test of gel-clot method in Appendix XII E). Each lot of sets shall bear a label with the product license number, lot number, expiry date and manufacturer. Before use, the blood bag shall be checked one by one, and the damaged ones and those with a leakage shall not be used.

(2) The anticoagulant solution shall be sterilized and free from pyrogen, preservative or antibiotics. A portion of 4% (g/ml) of sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) for injection, pH 7.2-7.6, or other suitable anticoagulants can be used. The sets with sterilized anticoagulant shall be sampled proportionally for checking the filling quantity of the anticoagulant, and the mean error shall be not more than 5%. Inspect the transparency of anticoagulant bag by bag before blood collection, and those with foreign matters or turbidity shall not be used. The bacterial endotoxin content shall be less than 5.56 EU/ml determined by the limit test of gel-clot method in Appendix XII E. Each lot of anticoagulant shall bear a label with product lot number, license number, validity period and manufacturer.

(3) The quality of sodium chloride injection shall accord with the requirements of the current Chinese Pharmacopoeia, and each lot of the solution shall bear a label with product license number, lot number and manufacturer. The sodium chloride injection used in plasma collection must be used by the principle of one bottle of the solution for one person exclusively at one time.

3. Plasmapheresis procedure

(1) Donors shall be identified and checked with his or her Certificate for Blood Donation before donation.

(2) Physical examination and laboratory testing shall be carried out as required, and only the qualified can be accepted for donation.

(3) Plasma shall be collected by plasmapheresis device.

(4) The volume of each donation shall be not more than 580 ml (containing anticoagulant, and it shall not exceed 600 g in terms of conversion from volume ratio to mass ratio).

(5) The sets (including syringe and the apparatus) used in plasmapheresis for plasma collection shall be disposable, and sterilized and destroyed after use immediately.

4. Donation frequency and volume limitation

The plasma volume collected from one donor shall be not more than 12000 ml each year and not more than 1200 ml each month, and the interval between two donations shall be not less than 2 weeks. Each donor should have a card of record bearing the dates of all donations, and the stipulation for the interval between donations shall be strictly followed, and it is not allowed to shorten the interval between donations or collect

plasma frequently.

5. Tests on plasma

Each bag of plasma shall be subject to the following tests.

(1) Appearance

It should be light yellow, yellow or light green in colour; no chyle, no fibrin separating out, no hemolysis or discernible foreign matters shall be found. The frozen plasma should present an intact form and firm shape.

(2) Protein content

The protein content shall be not less than 55 g/L determined with Biuret method (Appendix VI B, method 3) or by other approved methods.

(3) Alanine aminotransferase (ALT)

It shall be not more than 25 units detected by Reitman-Frankel method.

(4) Hepatitis B surface antigen

It shall be negative detected with the approved diagnostic kit.

(5) Syphilis

It shall be negative detected with the approved diagnostic kit.

(6) Anti-HIV-1/anti-HIV-2

It shall be negative detected with the approved diagnostic kit.

(7) Anti-HCV

It shall be negative detected with the approved diagnostic kit.

6. Package and label

(1) Package

Plastic bags for filling blood shall comply with the current national standards described in *GB-14232. The bags shall be intact. Plasma in the sample tube shall be completely the same as that in the bag.

(2) The content of label shall be complete and include donor's name, card number, blood group, plasma serial number, date of blood collection, weight of plasma and the name of plasmapheresis center.

III Plasma storage

The plasma shall be frozen within 6 hours after collection, and stored at or below -20°C . The storage period shall not exceed 24 months, unless otherwise specified.

There shall be temperature records during storage of plasma. If the frozen plasma thaws for not more than 72 hours due to temperature rising by accident, the thawed plasma can still be used for the fractionation of albumin and immunoglobulins.

IV Plasma shipping

1. The liquid plasma shall be shipped at $2-8^{\circ}\text{C}$ and the frozen one shall be shipped at -15°C or below, and a temperature record shall be available during shipping.

2. Source plasma shall be well packed with an intact outer package so as to avoid damage during shipping. There shall be a packaging sheet, the laboratory finding sheet and the plasma samples for checking in each carton.

3. During shipping when the temperature rises by accident, the plasma can be dealt with according to the requirements described in Section III.

V Donor immunization for preparing specific immunoglobulins

1. Plasma

(1) The plasma shall be collected from the donors immunized with licensed vaccines or immunogens, such as hepatitis A, hepatitis B, adsorbed tetanus, and rabies vaccines, etc. The titer of the antibody against the corresponding immunogens in the plasma shall reach to the required level.

(2) The immune plasma from naturally infected donor whose antibody titer has reached to the required level can be used.

(3) The standards of qualified plasma for antibody titer of both single-donor plasma and pooled plasma shall be laid down, respectively, unless otherwise specified.

(4) The collection and quality of the plasma from the above donors shall accord with the requirements described in Section I to Section IV.

2. Donors

(1) The health criteria for donors shall meet the requirements described in Section I.

(2) The donors shall be informed in advance the details of the likelihood of systemic or local reactions after immunization. The immunization of donors requires an informed consent in a written form or signing up a contract.

3. Immunization of donors

(1) The vaccines or other immunogens used for donor immunization shall be approved. Every effort shall be made to use the minimum dose of immunogen and the least injections for immunization.

(2) Immunization with more than one kind of immunogens can not be conducted before the safety of such immunizations is demonstrated.

(3) The immunization schedule for the donor can be different from that for routine immunization. However it can not be adopted before the safety of such special schedule is demonstrated and approved by the NRA.

(4) In order to avoid unforeseen accidents, donors shall be observed for at least 30 minutes following each immunization to see whether an instant adverse reaction occurs.

(5) There must be specified requirements for the donors who will be immunized with erythrocytes, and the requirements must be approved.

* GB-14232: National standards of 14232.

Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics

The Requirement apply to the cell substrates that are used for the production and control tests of biologics covering the cells with a cell banking system and primary cell cultures. The cell substrates of this monograph refer to the continuous cell lines, diploid cells as well as primary cell cultures derived from human or animal sources and used for the production of biologics.

For the non-recombinant products, the cell substrates refer to the cultures of primary cells and the unmodified cell line/strain chosen for preparation of master cell bank. For the recombinant products, the cell substrates refer to the transfected cells containing the desired sequences, which have been cloned from a single cell progenitor. For the products from hybridomas, the cell substrates refer to the hybridoma cell line which is derived by fusion of the cell from a parental myeloma cell line with another parental cell.

I General requirements for cell substrates of cell banks

The cell lines/strains used for the production and control tests of biologics shall be fully characterized and be approved by the NRA and the following documents shall be available.

(I) History of cell line/strain

1. Source of cell line/strain

The source of the cells should be stated including the name of the supplier, the information about the species, age, sex and health status of the cell donor. Information obtained directly from the source laboratory is preferable. When this is not available, literature references may be utilized.

For human cell line/strain, the following characteristics of the original donor shall be stated: tissue or organ of origin, ethnic and geographical origin, age, sex and general physiological conditions.

For animal cell line/strain, the following characteristics of the original donor shall be stated: species, strains, breeding conditions, tissue or organ of origin, geographical origin, age and sex, the results of tests for pathogenic agents, and general physiological condition of the original donor.

2. Cultivation history of cell line/strain

The cultivation history of cell line/strain shall be documented.

The methods and procedures used for the isolation and cultivation *in vitro* of the cells as well as the procedures used to establish the cell line/strain

shall be described, such as the physical, chemical or biological procedures used, the added nucleotide sequences, the cell growth character and the composition of culture medium. The description of any genetic manipulation or method used in cell selection shall be provided. And the information regarding the identification and characterization of these cells and the test results for endogenous and adventitious agents shall be provided.

The composition of the culture medium shall be described in detail, especially for the information regarding the materials of human or animal origin such as serum, trypsin, protein hydrolysates, or other bioactive materials. The description of above compositions shall include the source, method for preparation and quality control, test results, and quality assurance.

(II) Establishment of cell banks

The establishment of cell bank system makes it possible that biological products are produced with the cells derived from the cell bank system, which have been well characterized, and proved consistency in quality and stability in continuous passage.

1. Source materials

The donors of all kinds of cells for establishment of cell bank shall comply with the relevant requirements for quality control of cells in Section I (III). Cells from neurological origin shall not be used in the manufacture of biological products.

The bovine serum used in the cultivation of cells shall be purchased from the area without prevalence of bovine spongiform encephalopathy. The quality of serum shall comply with the related requirements in Appendix XIII D.

The human serum shall not be used in the culture medium. If human albumin is required, the licensed product shall be used.

The trypsin used in the dispersion of cells shall be proved free from contamination of cultivable bacteria, fungi, mycoplasmas and infectious viruses especially those viruses, e.g. parvoviruses potentially carried by the animals from which the trypsin was derived.

Penicillin or β -lactam antibiotics shall not be present in the cultures used for the production of biologics. The chemicals used for preparation of any kinds of solutions shall comply with the requirements in Volume II of the *Chinese Pharmacopoeia* or other relevant national standards.

2. Requirements of practice for cell manipulation

The manipulation of cells shall comply with the *Chinese GMP for Pharmaceutical Products*. The personnel concerned shall take a physical examination periodically. In the production area, handling of cells or microorganisms that are not used in the production is prohibited. On the same working day, the workers are prohibited to handle or contact with infectious microorganisms or animals before handling cells.

3. Generation of cell banks

The cell bank system should consist of three tiers; i.e. primary cell bank (PCB), master cell bank (MCB) and working cell bank (WCB). For the introduced cells, two tiers of MCB and WCB are generally adopted. For some special cases, a single-tier of WCB may be used and shall be subject to the approval of the NRA.

(1) Primary cell bank (PCB)

A cell population, developed from a primary cell population, that is consistent in subculture, or a homogeneous cell population derived from cloning cultivation which is demonstrated, through quality control, to be suitable for production and quality control of biologics can be established as the primary cell bank.

Under specified conditions, a certain amount of the cell suspension of uniform composition is dispensed in containers in aliquots, stored in liquid nitrogen or at -130°C or below. The primary cell bank is used to prepare MCB.

(2) Master cell bank (MCB)

Master cell bank (MCB) is made of the cells from the PCB which are propagated up to an amount of cells and then pooled homogeneously and dispensed in aliquots in containers. The MCB shall be stored in liquid nitrogen or at -130°C or below. These cells must be tested according to the specified quality control requirements, and shall not be used as the master cell bank until all the control tests are satisfactory. The master cell bank is used to prepare WCB.

(3) Working cell bank (WCB)

Working cell bank (WCB) is made of the cells from the MCB which are propagated up to an amount of cells and then pooled homogeneously and dispensed in aliquots in containers. These containers shall be stored in liquid nitrogen or at -130°C or below. The frozen cells shall be at the passage level which, after recovery, shall be able to propagate sufficient cells for the production of one lot or one sub-lot of biologics. The passage level of the cells at this stage after propagation shall be within the approved passage limit level used for the production of biologics. The cells of the WCB qualified in control tests can only be used for the production of biologics, as required for quality control of cells in Section I (III).

4. Management of cell banks

The inventory ledger for every cell bank shall be set up including the storage location, the storage container's number, quantity of cell vials and its utilization. The label, stuck on each vial of every cell bank, shall bear the cell line/strain name, number of generations, vial number, the date of storage, and the storage container's number.

The survival rate of frozen cells shall be more than 90%. The frozen cells shall be recovered once at least for continuous passage till the cells show decrepit. During passage it is required to examine the cell growth status at different passage levels.

The MCB and WCB shall be stored separately. Cells not used for production shall be stored separately from those used for production.

(III) Quality control of cell banks

The characterization and testing of the cells in cell banks mainly include cell identity test, tests for the presence of exogenous and endogenous agents and test for tumorigenicity, etc. In some cases, the karyological examination may be carried out if necessary. All the tests above mentioned are applicable to the MCB and the WCB.

Generally, the laboratory for generating cell banks shall perform the overall tests on the MCB at least once, and the tests include those for identity, sterility, bacteria, fungi, mycoplasma, and adventitious viruses, etc.

The newly established WCB shall be tested according to the related requirements.

1. Identity test

The identity test shall be carried out on the cells from newly established cell line/strain, the cell banks (MCB and WCB) and the cell cultures at the end of production of biologics to reconfirm that the cells should be the same as the original and not contaminated by other kinds of cells. Any one of the following methods can be used: cytogenetics tests (e.g. for chromosomal markers), test for genetic markers (e.g. DNA finger printing, STR mapping, genomic dinucleotide repeats), immunological test (e.g. human leucocyte antigen and species specific antiserum) and biochemical determinations (e.g. isozyme analysis). Any one of above methods can be used with the consent of the NCL. The phenotypic combined with genotypic characterizations may be more useful for the identity.

2. Tests for the presence of bacteria and fungi

The test samples from the pooled supernatants of cell cultures shall comply with sterility test (Appendix VII A).

3. Test for the presence of mycoplasmas

The supernatant fluids of the cell cultures shall comply with the test for mycoplasmas (Appendix VII B).

4. Tests for the presence of adventitious and endogenous viruses

The tests on the cell line/strain shall be carried out for the presence of infectious viruses potentially existing in the species from which the cells are derived, and the possibly contaminated extraneous viruses due to accidental operations. The kinds of potentially contaminated virus to be tested and the choice of tests should depend upon the origin of cell species, origin of the tissue and the cell characteristics.

(1) Observation on cell culture and haemadsorption test

The sample from pooled cells is inoculated into at least six flasks or Petri dishes. The medium is replaced with maintenance medium when cells are

confluent. The cells shall remain normal in morphological appearance microscopically during 14 day observation.

Add 0.2%-0.5% of guinea pig red cells mixed with chicken red cells onto the cell sheet of one third of cell culture flasks or Petri dishes after at least 14 day incubation. Put the flasks or Petri dishes successively at 4-8°C for 30 minutes and then at 20-25°C for 30 minutes to observe haemadsorption by microscopy. Both results shall be negative.

The freshly collected red cells shall be stored at 2-8°C for not more than 7 days and the preservative solution for red cells shall not contain calcium or magnesium ion.

(2) Test for the presence of viral agents using different cell cultures

The cells derived from the MCB or the WCB are inoculated onto the following three kinds of cells: simian cell cultures, human diploid cell cultures, and the cell cultures of the same kind but not the same batch. At least 10^7 live cells or disrupted cells and the spent culture fluids shall be inoculated onto each kind of cell cultures, not less than two flasks for each. The amount of inoculum shall account for more than one fourth of that of the maintenance medium. The inoculated cultures

should be incubated for at least 14 days. On the 7th day, the supernatants from the two flasks are inoculated respectively onto cell cultures of the same kind of cells for one blind passage. After 7-day incubation, observe the CPE and the observation of the cell morphological appearance and the haemadsorption test shall be performed.

If the cells being tested are known to be capable of supporting the growth of human cytomegalovirus, the human diploid cell cultures inoculated with the tested sample shall be observed for at least 28 days and no CPE shall be found. At the same time the haemadsorption test shall be carried out and the result shall be negative.

(3) Tests for the presence of viral agents using animals and chicken embryos

The cells from MCB or WCB and the cells propagated to or beyond the maximum culture age *in vitro* used for the production of biologics shall be tested for the presence of adventitious viral agents with inoculation of animals. The tests in animals and chicken embryos for adventitious agents are listed in Table 1. More than 80% of the inoculated animals or chicken embryos shall remain healthy and survive the observation period. And the evaluation should be finalized with reference to other tests.

Table 1 Tests for the adventitious agents with animals and chicken embryos

Test in	Specification	Number	Route for inoculation	Cell concentration (viables/ml)	Dosage (ml)	Days of observation	Result
Suckling mice	Within 24 hours after birth	More than 10(2 nests)	i. c	$>10^7$	0.01	21	Healthy survival
			i. p		0.1		
Adult mice	15-20 g	10	i. c	$>2 \times 10^6$	0.03	21	Healthy survival
			i. p		0.5		
Chicken embryos*	9-11 days	10	Allantoic cavity	$>5 \times 10^6$	0.2	3-4	Hemagglutination of Allantoic fluid negative
Chicken embryos	5-6 days	10	Yolk sac	$>2 \times 10^6$	0.5	5	Survival
Guinea pigs	350-500 g	5	i. p	$>4 \times 10^5$	5.0	42	Healthy survival, no tuberculous lesions in autopsy
Rabbits	1.5-2.5 kg	5	s. c.	$>2 \times 10^5$	9.0	21	No abnormal findings, healthy survival
			i. d**		0.1×10		

* Hemagglutination test is performed with mixed red cells of guinea pigs and chicken at the end of the observation period.

** 0.1 ml is injected to each of 10 spots on the skin of an individual rabbit.

For newly established cell line/strain, the guinea pigs and rabbits shall be inoculated (see Table 1). Guinea pigs are mainly used for testing *Mycobacterium tuberculosis* in the cells, and the tuberculin test in guinea pigs shall be performed 4 weeks before inoculation, respectively, and the results shall be negative. The test in rabbits for the presence of B virus in the cells of simian origin may be replaced by a test in rabbit kidney-cell cultures.

(4) Tests for the presence of retroviruses and

other endogenous agents or viral nucleic acids

The cells from the MCB or the WCB and the cells propagated to or beyond the maximum culture age limit *in vitro* used for the production of biologics shall be tested for retroviruses by the following methods:

① Reverse transcriptase (RT) assay; The activity of reverse transcriptase (RT) in the supernatant of cell cultures shall be tested by a highly sensitive method, such as Product of Enhanced Reverse

Transcriptase assay (PERT assay) or other sensitive assays.

②Transmission electron microscopic (TEM) examination: The cells to be tested are harvested by centrifugation at low speed. Discard the supernatant, and there shall be 1×10^7 cells in the pellet in which the viability of cells shall be less than 99%. The cell pellet is fixed with a fixative and then stored at 4°C or embedded directly to make ultrathin sections which are stained on the bronze sieve and examined by transmission electron microscopy.

③Infectivity assay: The cells susceptible to retroviruses shall be inoculated with the cells to be tested. It should be necessary to use the cells which are even more sensitive to the virus possibly present in tested cells for the infectivity assay according to the species origins of the cells to be tested.

The above mentioned three methods are different in the specificity and sensitivity. So it is recommended to use different methods in coordination to test for the presence of retroviruses.

If the result of reverse transcriptase assay is positive, it is recommended to perform the TEM or infectivity assay to reconfirm or deny the presence of the infectious retroviruses particles.

The cell lines derived from murine origin and other rodents or their hybridoma cell lines may potentially carry retroviruses. Therefore, it is necessary to test for the presence of the specific retroviruses in human-murine hybridoma cell lines. The murine cell line which is used for the production of monoclonal antibody may not be tested for the specific retroviruses, but it is necessary to implement an additional procedure for removal/inactivation of viruses during the production process.

(5) Tests for selected adventitious viruses

The cells from the MCB or the WCB shall be tested for selected viruses depending on the species of animal from which the cell line/strain is derived and the original tissue of the cell line/strain.

The species-specific viruses present in murine cell lines may be detected by mouse, rat and hamster antibody production tests.

Human cell lines should be screened for human virus pathogens, such as Epstein-Barr virus, human cytomegalovirus, human retroviruses, hepatitis B and C viruses. Under certain conditions, specific testing for the presence of other transforming viruses, such as human papillomavirus, adenovirus and herpes simplex virus may be indicated. Appropriate techniques *in vitro* may be used for the detection of these viruses.

5. Tumorigenicity test

If the continuous cell lines have already been demonstrated to be tumorigenic such as BHK21, CHO and C127, or if the cells belong to tumorigenic ones, for example hybridism, it is not necessary to carry out the tumorigenicity test.

However, for some cell lines (e.g. Vero cells)

including those having been demonstrated to be non-tumorigenic within a certain passage level and those to be tumorigenic beyond the certain passage level, the tumorigenicity test must be performed. Human epithelial cell lines, diploid cell strains and all cell lines/strains used for the live virus vaccine production shall be tested for the tumorigenicity. A newly established cell line/strain shall be tested for tumorigenicity.

In some cases, the cells to be used in somatic cell therapy or gene therapy shall be tested for tumorigenicity.

The cells from the MCB or WCB propagated to or beyond the culture age limit *in vitro* for production shall be tested for tumorigenicity.

One of the following test methods for tumorigenicity can be used.

①Nude mice: The cells to be tested are suspended in a quantity of serum-free medium to make the final concentration of at least 5×10^7 cells/ml. The cell suspension of 0.2 ml is inoculated s.c. or i.m. into each of at least ten nude mice. HeLa cells or Hep-2 cells are used as the positive control and each of at least ten animals is inoculated with 0.2 ml of 10^6 cells by the same route as that for the cells to be tested. Human diploid cells may be used as the negative control. The negative control group consists of at least ten animals, each of which should be inoculated with 0.2 ml of 10^7 cells.

②New born mice (3-5 days old): Each of ten animals weighing 8-10 g is inoculated with 0.1 ml of anti-thymocyte serum or globulin on days 0, 2, 7 and 14 after birth. Then each animal is inoculated s.c. with 0.2 ml of 10^7 cells as above mentioned. The positive control group is set up, in which at least ten animals shall be inoculated.

Result evaluation

①The animals shall be observed and palpated at regular intervals for the formation of nodules at the site of injection. Any nodules formed shall be measured in two dimensions and the data of the measurements shall be recorded.

②The test for tumorigenicity is valid provided that at least nine out of ten animals in positive control group progressively grow tumors.

③Animals with progressively growing nodules or suspicious focus shall be observed for 1-2 weeks at least. Animals showing nodules which begin to regress during the period of observation shall be killed before the nodules are no longer palpable, and processed for histopathological examination.

④Among those without nodule formation, half of the animals shall be observed for 21 days and half for 12 weeks before they are killed and processed for histological examination. A necropsy shall be performed on each animal including examination for gross evidence of tumour formation in lymph nodes and various organs. If any suspicions, histopathological examination shall be performed, and there shall be no metastasis.

In addition to *in vivo* testing, several *in vitro* test

systems, e.g. colon formation in soft agar or growth in organ culture can be used for tumorigenicity assay, which is particularly applicable to continuous cell lines of non-tumorigenicity in animals at low passage levels.

(IV) Cell cultures for production of biologics

The requirements for source materials and cell manipulation shall comply with the requirements for the establishment of cell banks in Section I (II). The cells from one or more frozen vials of the WCB are taken, and pooled for propagation up to a passage level for the production of biologics. And the level shall not exceed the maximum passage limit level of the cells for the production. The propagated cells of the cell seed taken from the WCB must not be restored in the freezer for further production.

Age counting of cell culture *in vitro*

The age for diploid cells is counted in terms of cell population doublings. Taking the number of cells in a vessel as the base, each doubling shall be one generation, i.e. one vessel is transferred into two vessels (1 : 2 subculture ratio). The transferred cells that have grown into a confluent sheet in these two vessels shall be regarded as one generation; one into four vessels (1 : 4) shall be two generations; and one into eight vessels (1 : 8) shall be three generations. The age of the cells for the production of biologics shall be limited to the early two-thirds of the life span.

The passage for continuous cell line is performed by dilution method, and each transfer is a passage.

II Specified requirements for continuous cell line

Continuous cell lines are generally derived from tumor tissues of human or animal origin, or from the passage or transformation of normal tissues. The cells can be cultivated in suspension or on carriers for production on a large scale. These cells can be propagated indefinitely but the tumorigenicity can be enhanced after certain passages. Therefore the continuous cells for the production of biologics shall be tested rigidly. The characterization of cell banks shall be carried out according to the requirements for quality control on cells in Section I (III). The requirements for the cell cultures during the production are as follows:

1. Cell passage level used for production of biologics
The number of cell passages of the continuous cell line used for production shall be defined. The maximum of cell passage level used for the production shall be approved.

2. Test for adventitious viruses at the end of production

For virus vaccines, the control of cell cultures shall be tested at the end of the production for haemadsorbing viruses according to the requirements for observation on cell morphological appearance

and haemadsorption test in Section I (III) 4 (1). If multiple harvest pools are prepared at different times, the control of cell cultures shall be tested at the time of the collection of each pool.

III Specified requirements for human diploid cell strains

The following documents are required for the establishment of new cell strain: the age and sex of the fetus used for the establishment of cell strain and the reason for the termination of pregnancy; the age, occupation and health conditions (a certificate of good health signed by physician, free from potential infectious diseases and hereditary defects) of the fetus' parents. There must be a report of investigation to demonstrate that the preceding three generations of the parents shall be evidently free from hereditary defects.

For human diploid cell strain, during the early stage of passages, an appropriate generation level of cells (2-8 generations) shall be selected to propagate up to a sufficient amount of cells for the preparation of cell suspension, which is dispensed into containers in aliquots, and stored in liquid nitrogen or at -130°C or below. It can only be defined as the primary cell bank when qualified in overall control tests, and used for preparing the master cell bank.

1. Examination on chromosomes and criteria for judgment

A newly established diploid cell strain and its cell banks must be examined for chromosomal characterization.

If cell banks are established from the identified human diploid cell lines (such as WI-38, MRC-5, 2BS, KMB17), the chromosomal recharacterization is not required unless the cells have been genetically modified.

(1) Examination on chromosomes

Examination on chromosomes shall be carried out every 8-12 generations during the course of establishing new cell strain. There shall be at least 4 times of results of chromosome examinations throughout the entire life span of the cell strain during continuous cultivation.

The number, morphology and structure of the chromosomes of at least 1000 metaphase cells shall be examined randomly, and the record shall be kept for rechecking. Photomicrography shall be performed for at least 50 metaphase cells and the karyotype analysis be made. At the same time, the frequency of polyploidy among 500 metaphase cells shall be investigated and recorded.

The chromosome slides are prepared by using the cultures of the mixed cells taken from different culture flasks of the same generation. The slides after examination shall be retained for rechecking. The techniques of G banding and Q banding can be used to examine the chromosome band types of 50 metaphase cells. Photographs shall be taken to

make band type analysis.

(2) Criteria for judgment

Examine the abnormality rate among 1000 and 500

metaphase cells, respectively; the upper limits for qualification are shown in Table 2 (the limit of 90% confidence, Poisson method).

Table 2 Upper limits for cells with abnormal chromosomes

Items of abnormality	Number of cells investigated		
	1000	500	100
Chromosomal monomer and chromosome breakage	47	26	8
Abnormal structure	17	10	2
Hyperdiploid	8	5	2
Hypodiploid*	180	90	18
Polyploid**	30	17	4

* If the hypodiploid exceeds the upper limit, which may result from the loss of chromosomes caused artificially during slide preparation, the counting shall be repeated with the specimen of the same batch.

** The metaphase cell with more than 53 chromosomes is defined as a polyploid.

2. Tests for the presence of bacteria and fungi

The cell cultures shall be tested for sterility every 8-12 generations (Appendix XII A).

3. Test for the presence of mycoplasmas

The cell cultures shall be tested for mycoplasmas every 8-12 generations (Appendix XII B).

4. Tests for selected viruses

During the passage of diploid cell strain the tests shall be carried out at least at two different passage levels for the presence of inclusion bodies, hepatitis B and C viruses, EB virus and HIV, and the electron microscopic examination be carried out, and all the results shall be negative.

5. Tumorigenicity test

The tumorigenicity test shall be carried out once every 8-12 generations according to the requirements for the tumorigenicity test in Section I (III) 5. The results shall be negative.

6. Tests on the cell cultures during production of biologics

(1) Examination on chromosomes

The usefulness of chromosomal characterization depends on the nature of the biological products and the manufacturing process. In general, products that might contain live cells or be insufficiently purified in the downstream will require chromosomal characterization and evaluation of the cell line (see the requirements for examination on chromosomes and control standards in Section III 1). No recharacterization of the karyology of cell substrates will be required if the established human diploid cells are used in production.

(2) Identity test

The identity test shall be carried out according to the requirements for the identity test on cells in Section I (III) 1.

(3) Tests for the presence of bacteria and fungi

It shall comply with the tests for sterility (Appendix XII A).

(4) Tests for the presence of mycoplasmas

It shall comply with the test for mycoplasmas (Appendix XII B).

(5) Tests for the presence of adventitious agents

For virus vaccines, on the same day of inoculating virus or at the last time of inoculating virus to the cell cultures during continuous passage of the virus, 2%-5% of the cell cultures uninoculated shall be reserved as the cell control after replacement of maintenance medium. The control cell cultures shall be incubated under the same condition as that of the virus-inoculated cultures and observed for 2 weeks. The tests for the presence of adventitious agents shall be carried out according to the requirements for observation on cell culture and haemadsorption test, and the tests for the presence of viral agents using different cell cultures in Sections I (III) 4 (1) and (2).

IV Specified requirements for the recombinant cells

The recombinant cell line is obtained through r-DNA technology and contains the desired gene sequence. Information regarding the methodologies utilized in developing the cell line shall be provided, for example, cell fusion, transfection, selection, colony isolation, cloning, gene amplification and the adaptation to specific culture conditions or media. The characterization of the cell bank shall be carried out according to the requirements for quality control of cells in Section I (III). In addition, the following tests shall be preformed.

1. Cell substrate stability

The manufacturer shall demonstrate and provide the information on the stability of the desired gene in the cell line used for production, including the genetic stability of the recombinant cell line, the expression stability of the desired gene, the consistency in the production of the intended product and the retention of production capacity of the cell line during storage under defined conditions.



2. Identity test

The desired gene or the intended protein shall be examined in addition to the test for identity of cells described in Section I (III) 1.

3. Tests for adventitious viral agents in the products of recombinant cells

The cell lysates or unprocessed bulk and culture medium shall be tested for the adventitious and endogenous viruses according to the requirements for observation on cell culture and haemadsorption test, and the tests for the presence of viral agents using different cell cultures in Sections I (III) 4 (1) and (2).

V Requirements for primary cells

The primary cells shall be derived from the normal tissues of viscera or embryos of healthy animals, including monkey kidney, hamster kidney, gerbil kidney, rabbit kidney, dog kidney, chicken embryos, quail embryos and other normal tissues. The primary cell cultures are prepared from the dispersed cells obtained by the trypsinization of normal tissues. It is unlikely to set up the cell bank system for the primary cells for which the characterization in advance is impossible. Therefore only the primary cells or those within a few passages shall be used for the production of biologics. In view of the difference between primary cell cultures and continuous cell lines, the management and operation measures shall be specified for primary cells to ensure the quality of biologics.

(I) Source of animal tissues and other materials

1. Source of animal tissues

It shall comply with the relevant requirements in the General Notices.

There shall be distinct requirements for the health status and cleanliness level of various animals.

2. Monkeys used for production of biologics and quality control

African green monkey and rhesus monkey are mostly used and the latter is mainly used in China. The monkeys shall be healthy and raised in cages or in small groups. The quarantine period shall be at least 6 weeks before using the animals to prepare cells. During the quarantine period, if any monkey shows signs of ill health or new monkeys get in, the quarantine shall be carried out again. The newly introduced monkeys shall be subject to tuberculin test and the test for the presence of simian herpes virus type 1 (B virus). Fetal monkey kidney can be used for production and the pregnant monkey must be quarantined

before taking out the fetus.

(II) Tests on primary cell cultures

The animals used for preparing cells shall be normal in autopsy. The extracted organs that are intended to prepare cells shall be normal, otherwise it shall not be used.

1. Tests of source materials for cell cultures and operations of cell cultures

The tests of source materials for cell cultures and operations of cell cultures shall comply with the requirements for selection of source materials and the requirements of practice for cell manipulation in Sections I (II) 1 and 2.

2. Tests on cell cultures

(1) Cell morphology examination

Before used in production or inoculated with virus, the cell cultures shall be examined visually and microscopically. There shall be no any suspicions, abnormalities or cytopathic effects and, if any, it shall not be used for production.

(2) Tests on control cells

A portion of 5%-10% (or at least 500 ml) of cell suspension of each batch of cell cultures shall remain uninoculated with virus. The cell concentration and treatment of the cell suspension shall be the same as that for the production and the cell suspension shall be reserved and observed for at least 14 days. The following tests shall be carried out and the results shall be negative.

① Tests for the presence of bacteria and fungi

The tests shall comply with the test for sterility (Appendix XII A).

② Test for the presence of mycoplasmas

The test shall comply with the test for mycoplasmas (Appendix XII B).

③ Tests for the presence of adventitious viruses

At the end of the observation period, cells shall be tested for the adventitious viruses according to requirements for observation on cell culture and haemadsorption test, and the tests for the presence of viral agents with different cell cultures in Sections I (III) 4 (1) and (2).

④ Tests for the presence of selected viruses in primary cell cultures

The primary monkey kidney cell cultures shall be tested for SV40, simian immunodeficiency virus and B virus emphatically by using Vero cells, primary green monkey kidney cells or rabbit kidney cells. The primary hamster kidney cell cultures shall be tested with BHK21 cell cultures to observe the cell morphology, if any suspicions, one blind passage on the same cells for further observation is required.

MONOGRAPHS

Contents of Monographs

I Biologics for Prophylaxis

Typhoid Vaccine	29
Typhoid and Paratyphoid A Combined Vaccine	32
Typhoid and Paratyphoid A & B Combined Vaccine	34
Vi Polysaccharide Typhoid Vaccine	36
Dysentery Vaccine (Live) of <i>S. flexneri</i> and <i>S. sonnei</i> , Oral	39
Group A Meningococcal Polysaccharide Vaccine	42
Leptospira Vaccine	45
Plague Vaccine (Live) for Percutaneous Scarification	48
Anthrax Vaccine (Live) for Percutaneous Scarification	51
Brucellosis Vaccine (Live) for Percutaneous Scarification	54
BCG Vaccine for Intradermal Injection	57
Diphtheria and Pertussis Combined Vaccine, Adsorbed	60
Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed	62
Diphtheria, Tetanus and Acellular Pertussis Combined Vaccine, Adsorbed	67
Tetanus Vaccine, Adsorbed	71
Diphtheria Vaccine, Adsorbed	75
Diphtheria Vaccine for Adults and Adolescents, Adsorbed	78
Diphtheria and Tetanus Combined Vaccine, Adsorbed	80
Diphtheria and Tetanus Combined Vaccine for Adults and Adolescents, Adsorbed	82
Japanese Encephalitis Vaccine, Inactivated	84
Japanese Encephalitis Vaccine, Live	88
Haemorrhagic Fever with Renal Syndrome (Type I) Vaccine, Inactivated	91
Haemorrhagic Fever with Renal Syndrome (Type II) Vaccine, Inactivated	94
Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine, Inactivated	97
Rabies Vaccine (Vero Cell) for Human Use	100
Rabies Vaccine (Vero Cell) for Human Use, Freeze-dried	103
Rabies Vaccine (Hamster Kidney Cell) for Human Use	107
Measles Vaccine, Live	110
Rubella Vaccine (Human Diploid Cell), Live	113
Rubella Vaccine (Rabbit Kidney Cell), Live	116
Mumps Vaccine, Live	119
Measles and Mumps Combined	

Vaccine, Live	122
Influenza Vaccine (Whole Virion), Inactivated	124
Hepatitis B Vaccine Made by Recombinant DNA Techniques in Yeast	127
Hepatitis B Vaccine Made by Recombinant DNA Techniques in CHO Cell	130
Hepatitis A Vaccine, Live	133
Hepatitis A (Live) Vaccine, Freeze-dried	136
Poliomyelitis Vaccine in Dragee Candy (Human Diploid Cell), Live	138
Poliomyelitis (Live) Vaccine (Monkey Kidney Cell), Oral	142
Poliomyelitis Vaccine in Dragee Candy (Monkey Kidney Cell), Live	146

II Biologics for Therapeutic Use

Diphtheria Antitoxin	150
Diphtheria Antitoxin, Freeze-dried	151
Tetanus Antitoxin	153
Tetanus Antitoxin, Freeze-dried	155
Gas-gangrene Antitoxin (Mixed)	157
Gas-gangrene Antitoxin (Mixed), Freeze-dried	159
Botulinum Antitoxins	160
Botulinum Antitoxins, Freeze-dried	162
<i>Agkistrodon halys</i> Antivenin, Equine	164
<i>Agkistrodon acutus</i> Antivenin, Equine	166
<i>Bungarus multicinctus</i> Antivenin, Equine	168
<i>Naja naja (atra)</i> Antivenin, Equine	170
Anthrax Antiserum	172
Rabies Antiserum	173
Human Albumin	175
Human Albumin, Freeze-dried	178
Human Immunoglobulin	180
Human Immunoglobulin, Freeze-dried	181
Human Hepatitis B Immunoglobulin	183
Human Hepatitis B Immunoglobulin, Freeze-dried	185
Human Rabies Immunoglobulin	186
Human Rabies Immunoglobulin, Freeze-dried	188
Human Tetanus Immunoglobulin	190
Human Tetanus Immunoglobulin, Freeze-dried	192
Human Immunoglobulin (pH4) for Intravenous Injection	193
Human Immunoglobulin (pH4) for Intravenous Injection, Freeze-dried	195
Human Immunoglobulin for Intravenous	

Injection	197
Human Coagulation Factor VIII	199
Human Fibrinogen	201
Human Prothrombin Complex	203
Anti-human T Lymphocyte Porcine Immunoglobulin	204
Anti-human T Lymphocyte Rabbit Immunoglobulin	207
Mouse Monoclonal Antibody Against Human CD3 Antigen of T Lymphocyte for Injection	209
Botulinum Toxin Type A for Injection	211
Recombinant Human Interferon $\alpha 1b$ for Injection	213
Recombinant Human Interferon $\alpha 1b$ Injection	215
Recombinant Human Interferon $\alpha 1b$ Eye Drops	217
Recombinant Human Interferon $\alpha 2a$ for Injection	219
Recombinant Human Interferon $\alpha 2a$ Injection	221
Recombinant Human Interferon $\alpha 2a$ for Injection (Yeast)	224
Recombinant Human Interferon $\alpha 2a$ Vaginal Suppository	226
Recombinant Human Interferon $\alpha 2b$ for Injection	228
Recombinant Human Interferon $\alpha 2b$ Injection	230
Recombinant Human Interferon $\alpha 2b$ for Injection (<i>P. putida</i>)	232
Recombinant Human Interferon $\alpha 2b$ Injection (<i>P. putida</i>)	235
Recombinant Human Interferon γ	

for Injection	237
Recombinant Human Interleukin-2 for Injection	239
Recombinant Human Erythropoietin for Injection (CHO Cell)	242
Recombinant Human Erythropoietin Injection (CHO Cell)	245
Recombinant Human Granulocyte Colony-stimulating Factor Injection	247
Recombinant Human Granulocyte/ Macrophage Colony-stimulating Factor for Injection	249
Recombinant Streptokinase for Injection	251
Recombinant Bovine Basic Fibroblast Growth Factor for External Use, Liquid	254
Recombinant Bovine Basic Fibroblast Growth Factor for External Use	256
Recombinant Bovine Basic Fibroblast Growth Factor Eye Drops	258
Recombinant Human Epidermal Growth Factor for External Use	260
Recombinant Human Epidermal Growth Factor Derivative for External Use, Liquid	262

III Diagnostic Reagents for *in vivo* Test

Purified Protein Derivative of Tuberculin (TB-PPD)	265
Purified Protein Derivative of BCG (BCG-PPD)	267
Purified Protein Derivative of Brucellin (BR-PPD)	270
Schick Test Toxin	273

I Biologics for Prophylaxis

Typhoid Vaccine

Typhoid vaccine is a suspension of *Salmonella typhi* made by cultivation, inactivation with formaldehyde and dilution with PBS. It is used to prevent typhoid fever.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The bacterial strains for production shall be strains of *Salmonella typhi*.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lot

The subculture of the seed from master seed lot shall not exceed five passages; the subculture of the seed from working seed lot inoculated onto a production medium shall not exceed five passages.

2.1.4 Control tests on seed lots

2.1.4.1 Cultural characteristics and microscopic examination of stained smears

The bacterial seed to be tested shall be inoculated onto broth agar, Martin agar or other appropriate media, and incubated at 37°C for 18-20 hours. The colonies shall be smooth, round, colourless, translucent, humid and with regular border. The cultures shall be Gram-negative rods by microscopic examination of stained smears.

2.1.4.2 Biochemical characteristics

The cultures shall ferment glucose, maltose, and mannitol with production of acid but not gas. They shall not ferment lactose or sucrose

(Appendix XIV), and shall be oxidase-negative.

2.1.4.3 Serological characteristics

(1) Slide agglutination test

The fresh culture of the bacterial seed to be tested shall agglutinate strongly with anti-Vi and anti-H-d reference sera (+++ or more), but show little or no agglutination with anti-O-9 reference serum.

(2) Quantitative agglutination test

The fresh culture of the bacterial seed to be tested shall be diluted with PBS to a suspension containing 6.0×10^8 bacteria/ml, and a quantitative agglutination test shall be performed on the suspension with reference sera for typhoid. The suspension shall be mixed with the serum, allow to stand at 35-37°C overnight, and the results shall be observed visually. The highest dilution of serum which gives an agglutination (+) is regarded as agglutination titer, and the agglutination titer shall be not less than half of the original titer of the serum.

2.1.4.4 Virulence test

The bacterial culture after incubation on agar at 35-37°C for 12-16 hours shall be diluted with physiological saline to suspensions containing 6.0×10^8 , 3.0×10^8 , 1.5×10^8 and 7.5×10^7 bacteria/ml, respectively (dilution may change according to the virulence of the strain). Inject i. p. 0.5 ml of each dilution of suspension into each of at least five mice, weighing 14-16 g, and observe the mice for 3 days. The minimum dose of suspension that kills all of mice is regarded as 1 MLD. One MLD shall contain not more than 1.5×10^8 bacteria.

2.1.4.5 Toxicity test

The bacterial culture after incubation on agar at 35-37°C for 18-20 hours shall be suspended in PBS, and heated at 56°C for 1 hour (or inactivated by other methods). No preservative shall be added. The suspension qualified in bactericidal test shall be diluted to the concentrations of 6.0×10^9 , 3.0×10^9 and 1.5×10^9 bacteria/ml, respectively. Inject i. p. 0.5 ml of each concentration of suspension into each of five mice, weighing 15-18 g, and observe the mice for 3 days. All of the mice inoculated with 7.5×10^8 bacteria shall survive and three of five mice receiving 1.5×10^9 bacteria may die.

2.1.4.6 Immunity test

The preservative-free bacterial suspension inactivated by heating at 56°C for 30 minutes (or other methods) is diluted to a concentration of 2.5×10^8 bacteria/ml. Each of at least thirty mice, weighing 14-16 g, shall be given two injections of 0.5 ml of the suspension by subcutaneous route at an interval of 7 days. The mice shall be challenged with virulent bacteria 9-11 days after the last injection. Each mouse in immunized group is challenged i. p. with 0.5 ml containing 1 MLD of virulent bacteria, and each mouse in three control groups (at least five mice in each group) is challenged i. p. with 0.5 ml of 2, 1, and 1/2 MLD of virulent bacteria, respectively. The mice in immunized group and control groups have the same feeding conditions or the same body weights. The mice shall be observed for 3 days. The mice in control groups challenged with 2 MLD and 1 MLD shall totally die, and those with 1/2 MLD shall partially die, while at least 70% of mice in immunized group shall survive.

Immunity test may also be performed by the method of LD₅₀ challenge. The preservative-free bacterial suspension inactivated by heating at 56°C for 30 minutes (or other methods) shall be diluted to a concentration of 2.5×10^8 bacteria/ml. Each of at least thirty mice, weighing 14-16 g, shall be given two injections of 0.5 ml of the suspension by subcutaneous route at an interval of 7 days. The mice shall be challenged with virulent bacteria 9-11 days after the last injection. The lawn after incubation for 12-16 hours shall be diluted with broth pH 7.2-7.4 or physiological saline to an appropriate concentration and used for challenge. Each mouse in immunized group shall be infected with more than 100 LD₅₀ of virulent bacteria, and each mouse in three to four control groups (at least five mice in each group) injected with various doses of virulent bacteria, respectively. The mice in immunized group and control groups have the same feeding conditions or the same body weights. Each mouse in both groups shall be challenged i. p. with 0.5 ml of virulent bacteria and observed for 3 days, and LD₅₀ shall be determined. At least 70% of mice in immunized group shall survive. The reference vaccine shall be used as a control simultaneously.

2.1.4.7 Antigenicity test

Each of at least three healthy rabbits, weighing about 2 kg, shall be given three injections of 0.5 ml of the preservative-free bacterial suspension inactivated by heating at 56°C for 30 minutes (or other methods), by intravenous route at intervals of 7 days. First dose contains 7.0×10^8 bacteria, second dose 1.4×10^9 bacteria, and third dose 2.1×10^9 bacteria. Collect blood sample and perform a quantitative agglutination test 10-14 days after the last injection. The serum agglutination titers of two-thirds of rabbits shall be not less than 1 : 12800.

2.1.5 Storage of seed lot

Primary seed lot and master seed lot shall be lyophilized and preserved at 2-8°C; working seed lot shall be preserved in appropriate medium at 2-8°C.

2.2 Bulk

2.2.1 Working seed lots for production

After all characteristics are examined as qualified, the bacterial seed derived from working seed lot shall be inoculated onto a modified semisynthetic medium or other appropriate media to prepare working seed for production.

2.2.2 Production medium

Media pH 7.2-7.4, such as Martin agar, broth agar or other approved media shall be used for production.

2.2.3 Inoculation and cultivation

After inoculation on medium by method of smearing, the cultures shall be cultivated at 35-37°C for 18-24 hours.

2.2.4 Harvest

The bulk shall be prepared from the collected bacterial lawn by suspending in PBS, and tested for bacterial purity. Samples taken from each bottle of bulk shall be inoculated onto two tubes of agar slant, and incubated at 35-37°C for 2 days and at 24-26°C for one day, respectively. If any contaminating microorganisms are found, the bulk shall be discarded.

2.2.5 Killing

The bulk qualified in bacterial purity test shall be inactivated by addition of formalin to a final concentration of 1.0%-1.2% and placed at 37°C for a period not exceeding 7 days, then stored at 2-8°C.

2.2.6 Sterility test

After killing of organisms, the samples taken from bulk shall be inoculated onto thioglycollate medium containing no agar and common agar slant respectively, and incubated at 35-37°C for 5 days. If growth of *Salmonella typhi* is found, retest shall be performed with double amount of the medium. If any contaminating microorganisms are found, the bulk shall be discarded.

2.2.7 Pooling

The bulks qualified in sterility test, from the same strain or those with the same production date, shall be pooled respectively, following removal of agar and other impurities. Phenol or other appropriate preservatives, at a final concentration of not more than 3.0 g/L, shall be added to the pooled bulk, which shall be stored at 2-8°C.

2.2.8 Control tests on bulk

See Section 3.1.

2.2.9 Storage and storage period

The bulk shall be stored at 2-8°C. The duration from harvest of bulk to vaccine dilution shall be not less than 4 months. The storage period is 30

months starting from the date of harvest.

2.3 Final bulk

2.3.1 Formulation

Before dilution, the bulks prepared from different bacterial strains shall be mixed with the equal number of bacteria. The bacterial number of each strain is permitted to increase or decrease within the range of 40% but the total number of bacteria shall remain unchanged. The bulk shall be diluted with PBS containing not more than 3.0 g/L of phenol or other appropriate preservatives. The concentration following dilution shall be 3.0×10^8 bacteria/ml.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

5 ml per container. Each single human dose is 0.2-1.0 ml (depending on the age of eligible and number of injections) containing 6.0×10^7 - 3.0×10^8 bacteria.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Microscopic examination of stained smears
The Gram-negative rods shall be observed. No contaminating microorganisms shall be found.

3.1.2 Agglutination test

A quantitative agglutination test shall be performed on the bulk with corresponding serum. The agglutination titer shall be not less than half of the titer of the original serum.

3.1.3 Bacterial content

The bacterial content shall be determined against the National Standard of Bacterial Opacity.

3.1.4 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.5 Immunity test

The bulk qualified in sterility test shall be tested for immunity. The batches to be sampled for the test shall be not less than one-fifth of the total production batches. See Section 2.1.4.6. At least fifteen mice shall be used in each group. The test is qualified if not less than 60% of mice in immunized group survive.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

A slide agglutination test shall be performed with the corresponding serum, and distinct agglutination shall be observed.

3.3.2 Inspection on final containers

The vaccine shall be a milky-white suspension free of foreign matters and clumps not dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.8-7.4 (Appendix V A).

3.3.3.2 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.3.4 Bacterial morphology and purity

The bacteria shall be Gram-negative rods by microscopic examination of stained smear. At least ten fields shall be observed. On an average, there shall be not more than ten atypical bacteria (thready, gross or abnormally stained bacteria) in each field, and free from contaminating microorganisms.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix X F). The injecting dose of the product for each guinea pig shall be 1.5 ml.

4 Storage, shipping, and validity period

Store and ship at 2-8°C, protected from light. The validity period is 18 months starting from the date of filling of final product. The validity period shall be shortened correspondingly if the bulk has been stored for more than 12 months before dilution. The total validity period shall not exceed 30 months starting from the date of harvest.

5 Package inserts

Directions for Use of Typhoid Vaccine

[Drug name]

Adopted name: Typhoid Vaccine

[Constituents and characters]

Typhoid vaccine is a suspension of *Salmonella typhi* made by cultivation, inactivated with formaldehyde and diluted with PBS. The final product is a milky-white suspension containing phenol as a preservative.

[Eligibles]

Military personnel, port and railway workers, sewage and rubbish disposal workers, caterers, medical and antiepidemic personnel, boat dwellers,

and residents of endemic areas of typhoid fever.

[Function and usage]

The vaccine can induce immune response in recipients following immunization. It is used to prevent typhoid fever.

[Specifications]

5 ml per container. Each single human dose is 0.2-1.0 ml (depending on the age of eligible and the number of injections) containing 6.0×10^7 - 3.0×10^8 bacteria.

[Administration and dosage]

(1) The vaccine should be injected s. c. at deltoid insertion area of the lateral upper arm.

(2) Primary immunization; three injections shall be given at intervals of 7-10 days. The dosages are as follows:

Children at 1-6 years of age; first injection 0.2 ml, second injection 0.3 ml, and third injection 0.3 ml.

Children at 7-14 years of age; first injection 0.3 ml, second injection 0.5 ml, and third injection 0.5 ml.

Persons over 14 years of age; first injection 0.5 ml, second injection 1.0 ml, and third injection 1.0 ml.

The dosage for booster is the same as that for the third injection.

[Adverse reactions]

Erythema and swelling may occur at the injection site. Systemic manifestations may include chill, fever and headache, which can be relieved spontaneously.

[Contraindications]

The vaccine should not be administered to the subjects with the following conditions:

- (1) Fever, serious hypertension, cardiac, hepatic, and renal diseases, and active tuberculosis.
- (2) Pregnancy, menstruation and lactation.
- (3) A history of allergic reactions.

[Precautions]

(1) Shake container before use. Do not use the vaccine if any leakage of container, foreign matters or clumps not dispersed on shaking are found, or the product has been frozen.

(2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(3) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Typhoid and Paratyphoid A Combined Vaccine

Typhoid and paratyphoid A combined vaccine is a suspension of *Salmonella typhi* and *Salmonella paratyphi* A made by cultivation, inactivation with formaldehyde and dilution with PBS. It is used to prevent typhoid fever and paratyphoid A fever.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Monovalent bulk before mixing

2.1.1 Bulk typhoid vaccine shall comply with the requirements given in Sections 2.1-2.2 and 3.1 of the requirements for Typhoid Vaccine.

2.1.2 Bulk paratyphoid A vaccine shall comply with the requirements given in Sections 2.1-2.2 and 3.1 of the requirements for Typhoid Vaccine. The bacterial strain for production shall be *Salmonella paratyphi* A. The virulence of *Salmonella paratyphi* A shall be 1 MLD containing not more than 7.5×10^8 bacteria in Section 2.1.4.4. The concentration of *Salmonella paratyphi* A for immunization shall be 5.0×10^8 bacteria/ml, and at least 60% of mice in *Salmonella paratyphi* A suspension-immunized group shall survive the challenge in Section 2.1.4.6. The serum agglutination titer of rabbits immunized with *Salmonella paratyphi* A suspension shall be not less than 1 : 6400 in Section 2.1.4.7. Bulk paratyphoid A vaccine shall be inactivated by addition of formalin to a final concentration of 1.3%-1.5% in Section 2.2.5.

2.2 Final bulk

2.2.1 Formula

The final bulk shall contain 1.5×10^8 *Salmonella typhi*/ml and 1.5×10^8 *Salmonella paratyphi* A/ml.

2.2.2 Pooling and dilution

The bulk prepared from different bacterial species shall be mixed proportionally. The bacterial number of each species is permitted to increase or decrease within the range of 20% but the total

number of bacteria shall remain unchanged. The bulk prepared from different bacterial strains of the same species shall be mixed with the equal number of bacteria. The bacterial number of each strain is allowed to increase or decrease within the range of 40% , but the total number of bacteria shall remain unchanged. The vaccine shall be diluted with PBS containing not more than 3.0 g/L of phenol or other appropriate preservatives to a concentration of 1.5×10^8 *Salmonella typhi*/ml and 1.5×10^8 *Salmonella paratyphi A*/ml.

2.2.3 Control tests on final bulk
See Section 3.1.

2.3 Final product

2.3.1 Defining batches

The Requirements for Defining of Biologics shall apply.

2.3.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.3.3 Specifications

5 ml per container. Each single human dose is 0.2-1.0 ml (depending on the age of eligible and the number of injections) containing 3.0×10^7 - 1.5×10^8 *Salmonella typhi* and 3.0×10^7 - 1.5×10^8 *Salmonella paratyphi A*.

2.3.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.2 Control tests on final product

3.2.1 Identity test

A slide agglutination test shall be performed with the corresponding serum, and distinct agglutination shall be observed.

3.2.2 Inspection on final container

The vaccine shall be a milky-white suspension free of foreign matters and clumps not dispersed on shaking.

3.2.3 Chemical tests

3.2.3.1 pH

The pH shall be 6.8-7.4 (Appendix V A).

3.2.3.2 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.2.3.3 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.2.4 Bacterial morphology and purity

The bacteria shall be Gram-negative rods by microscopic examination of stained smear. At least

ten fields shall be observed. On an average, there shall be not more than ten atypical bacteria (thready, gross or abnormally stained bacteria) in each field, and free from contaminating micro-organisms.

3.2.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F). The injecting dose for each guinea pig shall be 1.5 ml.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 18 months starting from the date of filling of final product. The validity period shall be shortened correspondingly if the bulk has been stored for more than 12 months before dilution. The total validity period shall not exceed 30 months starting from the date of harvest.

5 Package inserts

Directions for Use of Typhoid and Paratyphoid A Combined Vaccine

[Drug name]

Adopted name: Typhoid and Paratyphoid A Combined Vaccine

[Constituents and characters]

The vaccine is a suspension made by cultivation of *Salmonella typhi* and *Salmonella paratyphi A*, inactivation with formaldehyde and dilution with PBS. It is a milky-white suspension containing phenol as a preservative.

[Eligibles]

Military personnel, port and railway workers, sewage and rubbish disposal workers, caterers, medical and antiepidemic personnel, boat dwellers, and residents of endemic areas of typhoid fever and paratyphoid A fever.

[Function and usage]

The vaccine is used to prevent typhoid fever and paratyphoid A fever.

[Specifications]

5 ml per container. Each single human dose is 0.2-1.0 ml (depending on the age of eligible and the number of injections) containing 3.0×10^7 - 1.5×10^8 *Salmonella typhi* and 3.0×10^7 - 1.5×10^8 *Salmonella paratyphi A*.

[Administration and dosage]

(1) The vaccine should be injected s. c. at deltoid insertion area of the lateral upper arm.

(2) Primary immunization: three injections shall be given at intervals of 7-10 days. The dosages are as follows:

Children at 1-6 years of age: first injection 0.2 ml, second injection 0.3 ml, and third injection 0.3 ml.

Children at 7-14 years of age: first injection 0.3 ml,

second injection 0.5 ml, and third injection 0.5 ml.

Persons over 14 years of age: first injection 0.5 ml, second injection 1.0 ml, and third injection 1.0 ml.

The dosage for booster is the same as that for the third injection.

[Adverse reactions]

Erythema and swelling may occur at the injection site. Systemic manifestations may include chill, fever and headache, which can be relieved spontaneously.

[Contraindications]

The vaccine should not be administered to the subjects with the following conditions:

- (1) Fever, serious heart diseases, hypertension, hepatic and renal diseases, and active tuberculosis.
- (2) Pregnancy, menstruation and lactation.
- (3) A history of allergic reactions.

[Precautions]

(1) Shake container before use. Do not use the vaccine if any leakage of container, foreign matters or clumps not dispersed on shaking are found, or the product has been frozen.

(2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(3) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Typhoid and Paratyphoid A & B Combined Vaccine

Typhoid and paratyphoid A&B combined vaccine is a suspension of *Salmonella typhi* and *Salmonella paratyphi* A&B made by cultivation, inactivation with formaldehyde and dilution with PBS. It is used to prevent typhoid fever and paratyphoid A&B fever.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Monovalent bulk before mixing

2.1.1 Bulk typhoid vaccine shall comply with the requirements given in Sections 2.1-2.2 and 3.1 of the Typhoid Vaccine.

2.1.2 Bulk paratyphoid A vaccine shall comply with the requirements given in Section 2.1.2 of the Typhoid and Paratyphoid A Combined Vaccine.

2.1.3 Bulk paratyphoid B vaccine shall comply with the requirements given in Sections 2.1-2.2 and 3.1 of the Typhoid Vaccine. The bacterial strain for production shall be *Salmonella paratyphi* B. The serum agglutination titer of rabbits immunized with *Salmonella paratyphi* B suspension shall be not less than 1 : 6400 in Section 2.1.4.7. Bulk paratyphoid B vaccine shall be inactivated by addition of formalin to a final concentration of 1.6%-2.0% in Section 2.2.5.

2.2 Final bulk

2.2.1 Formula

The final bulk shall contain 1.5×10^8 *Salmonella typhi*/ml, 7.5×10^7 *Salmonella paratyphi* A/ml and 7.5×10^7 *Salmonella paratyphi* B/ml.

2.2.2 Pooling and dilution

The bulk prepared from different bacterial species shall be mixed proportionally. The bacterial number of each species is permitted to increase or decrease within the range of 20% but the total number of the bacteria shall remain unchanged. The bulks prepared from different bacterial strains of the same species shall be mixed with an equal number of bacteria. The bacterial number of each strain is allowed to increase or decrease within the range of 40% but the total number of the bacteria shall remain unchanged. The vaccine shall be diluted with PBS containing no more than 3.0 g/L of phenol or other appropriate preservatives to a concentration of 1.5×10^8 *Salmonella typhi*/ml, 7.5×10^7 *Salmonella paratyphi* A/ml and 7.5×10^7 *Salmonella paratyphi* B/ml.

2.2.3 Control tests on final bulk

See Section 3.1.

2.3 Final product

2.3.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.3.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.3.3 Specifications

5 ml per container. Each single human dose is 0.2-1.0 ml (depending on the age of eligible and the



number of injections) containing 3.0×10^7 - 1.5×10^8 *Salmonella typhi*, 1.5×10^7 - 7.5×10^7 *Salmonella paratyphi* A and 1.5×10^7 - 7.5×10^7 *Salmonella paratyphi* B.

2.3.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.2 Control tests on final product

3.2.1 Identity test

A slide agglutination test shall be performed with the corresponding serum, and distinct agglutination shall be observed.

3.2.2 Inspection on final containers

The vaccine shall be a milky-white suspension free of foreign matters and clumps not dispersed on shaking.

3.2.3 Chemical tests

3.2.3.1 pH

The pH shall be 6.8-7.4 (Appendix V A).

3.2.3.2 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.2.3.3 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.2.4 Bacterial morphology and purity

The bacteria shall be Gram-negative rods by microscopic examination of stained smear. At least ten fields shall be observed. On an average, there shall be not more than ten atypical bacteria (thready, gross or abnormal stained bacteria) in each field, and free from contaminating micro-organisms.

3.2.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F). The injecting dose for each guinea pig shall be 1.5 ml.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 18 months starting from the date of filling of final product. The validity period shall be shortened correspondingly if the bulk has been stored for more than 12 months before dilution. The total validity period shall not exceed 30 months starting from the date of bulk harvest.

5 Package inserts

Directions for Use of Typhoid and Paratyphoid A & B Combined Vaccine

[Drug name]

Adopted name: Typhoid and Paratyphoid A & B Combined Vaccine

[Constituents and characters]

The vaccine is a suspension made by cultivation of *Salmonella typhi* and *Salmonella paratyphi* A&B, inactivation with formaldehyde and dilution with PBS. It is a milky-white suspension containing phenol as a preservative.

[Eligibles]

Military personnel, port and railway workers, sewage and rubbish disposal workers, caterers, medical and antiepidemic personnel, boat dwellers, and residents of endemic areas of typhoid fever and paratyphoid A&B fever.

[Function and usage]

The vaccine can induce immune response in recipients following immunization. It is used to prevent typhoid fever and paratyphoid A&B fever.

[Specifications]

5.0 ml per container. Each single human dose is 0.2-1.0 ml (depending on the age of eligible and the number of injections) containing 3.0×10^7 - 1.5×10^8 *Salmonella typhi*, 1.5×10^7 - 7.5×10^7 *Salmonella paratyphi* A and 1.5×10^7 - 7.5×10^7 *Salmonella paratyphi* B.

[Administration and dosage]

(1) The vaccine should be injected s. c. at deltoid insertion area of the lateral upper arm.

(2) Primary immunization: three injections shall be given at intervals of 7-10 days. The dosages are as follows:

Children at 1-6 years of age: first injection 0.2 ml, second injection 0.3 ml, and third injection 0.3 ml.

Children at 7-14 years of age: first injection 0.3 ml, second injection 0.5 ml, and third injection 0.5 ml.

Persons over 14 years of age: first injection 0.5 ml, second injection 1.0 ml, and third injection 1.0 ml.

The dosage for booster is the same as that for the third injection.

[Adverse reactions]

Erythema and swelling may occur at the injection site. Systemic manifestations may include chill, fever and headache, which can be relieved spontaneously.

[Contraindications]

The vaccine should not be administered to the subjects with the following conditions:

- (1) Fever, serious heart diseases, hypertension, hepatic and renal diseases, and active tuberculosis.
- (2) Pregnancy, menstruation and lactation.
- (3) A history of allergic reactions.

[Precautions]

(1) Shake container before use. Do not use the vaccine if any leakage of container, foreign matters or clumps not dispersed on shaking are found, or the product has been frozen.

(2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(3) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;

Address;

Zip code;

Tel;

Fax;

Web site;

Vi Polysaccharide Typhoid Vaccine

Vi polysaccharide typhoid vaccine is a purified Vi polysaccharide antigen extracted from the culture of *Salmonella typhi* and diluted with PBS. It is used to prevent typhoid fever.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The strain of bacterium used for production shall be *S. typhi* strain Ty2.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lot

The subculture of the seed from master seed lot shall not exceed five passages; the subculture of the seed from working seed lot inoculated into a fermentor shall not exceed five passages.

2.1.4 Control tests on seed lots

2.1.4.1 Cultural characteristics and microscopic examination of stained smears

The bacterial seed shall be inoculated onto broth agar, Martin agar or other appropriate media of pH 7.2-7.4, and incubated at 35-37°C for 16-20 hours. The colonies shall be smooth, round, colourless, translucent, humid and with regular border. The cultures shall be Gram-negative rods by microscopic examination of stained smears.

2.1.4.2 Biochemical characteristics

The cultures shall ferment glucose, maltose, and mannitol with production of acid but not gas. They shall not ferment lactose or sucrose (Appendix XIV) and shall be oxidase-negative.

2.1.4.3 Serological characteristics

(1) Slide agglutination test

The fresh cultures of the bacterial seed shall agglutinate strongly with anti-Vi and anti-H-d reference sera (+++ or more), but show little or no agglutination with anti-O-9 serum.

(2) Quantitative agglutination test

The fresh cultures of the bacterial seed shall be diluted with PBS to a suspension containing 6.0×10^8 bacteria/ml. The bacterial suspension shall be inactivated by adding formalin to a final concentration of 0.5%. Quantitative agglutination test shall be performed on the inactivated suspension or a live suspension with anti-Vi serum. The test shall also be performed on the bacterial suspension inactivated by heating at 100°C for 30 minutes with reference anti-O serum. The highest dilution of serum which gives an agglutination (+) is regarded as agglutination titer, and the agglutination titer shall be not less than half of the titer of the original serum.

2.1.5 Storage of seed lot

Primary seed lot and master seed lot shall be lyophilized and preserved at 2-8°C; working seed lot shall be preserved in appropriate medium at 2-8°C.

2.2 Bulk

2.2.1 Working seed lots for production

After all characteristics are examined as qualified, the bacterial seed derived from working seed lot shall be inoculated onto a modified semisynthetic medium or other appropriate media to prepare a quantity of working seeds for production.

2.2.2 Production medium

The modified semisynthetic medium or other approved media shall be used for production. The media shall be free from ingredients that may form precipitate upon addition of hexadecyltrimethylammonium bromide.

2.2.3 Cultivation

Inoculate the bacterial seed into the liquid medium in a fermentor. Take samples during cultivation and before killing and test for bacterial content,

and for purity by microscopic examination of Gram-stained smears. If any contaminating micro-organisms are found, the culture shall be discarded.

2.2.4 Killing

The cultures shall be harvested during the late logarithmic or early stationary phase. The harvested bacteria shall be killed by addition of formalin to a final concentration of 0.5%-2.0%. It is proper to ensure complete killing of bacteria without damage to its polysaccharide antigen.

2.2.5 Purification

2.2.5.1 Removal of nucleic acid

After killing the bacteria, centrifuge the cultures (a single harvest or pooled harvests), and collect the supernatant. To the supernatant, add hexadecyltrimethylammonium bromide and mix well to form a precipitate. Collect the precipitate by centrifugation. Dissolve the precipitate with water for injection, add a quantity of 1 mol/L sodium chloride (or 2 mol/L calcium chloride) solution, and shake or agitate for 1 hour to dissociate the polysaccharide from hexadecyltrimethylammonium bromide. Add ethanol to a final concentration of 25%, and allow to stand for 1-3 hours or overnight at 2-8°C. Centrifuge and collect the clear supernatant.

2.2.5.2 Precipitation of polysaccharide

To the above mentioned supernatant, add cold ethanol to a final concentration of 80%, mix well to precipitate the polysaccharide, and collect the precipitate by centrifugation. Wash the precipitate twice with absolute ethanol and acetone, respectively, allow to dry and obtain the crude polysaccharide. Store the crude polysaccharide at or below -20°C for purification.

2.2.5.3 Purification of polysaccharide

Dissolve the crude polysaccharide in 1/10 saturated neutral sodium acetate solution to a concentration of 5-20 mg/ml, then extract several times with twice its volume of cold phenol (dissolve 100 g crystalline phenol in 40 ml of 1/10 saturated sodium acetate). Collect the supernatant by centrifugation, and dialyse against 0.1 mol/L calcium chloride solution or other appropriate solutions. Other methods may be adopted to remove the endotoxic activity, if necessary. To the supernatant, add ethanol to a final concentration of 75%-80%. Collect the precipitated polysaccharide by centrifugation, and wash it with absolute ethanol and acetone for 2 times or more, respectively. After drying, dissolve the precipitate in sterile water for injection and prepare the bulk purified polysaccharide. Purification process shall be carried out at or below 15°C.

2.2.6 Control tests on bulk

Take samples from the bulk purified polysaccharide after sterilization by filtration. See Section 3.1.

2.2.7 Storage and storage period

The bulk shall be stored at or below -20°C. The storage period is 60 months starting from the date when crude polysaccharide is produced.

2.3 Final bulk

2.3.1 Formulation

The final bulk shall be prepared from a single batch or by pooling a number of single batches of bulk polysaccharide. It shall be diluted with sterile, pyrogen-free PBS (pH 6.5-7.5) and a quantity of preservative may be added. Each single human dose shall contain not less than 30 µg of polysaccharide.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

5 ml (10 human doses), 1 ml (2 human doses), or 0.5 ml (single human dose) per container. Each single human dose is 0.5 ml containing not less than 30 µg of Vi polysaccharide.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Identity test

Carry out the identity test by the method of double immunodiffusion (Appendix VIII C). The bulk Vi polysaccharide shall form an apparent precipitation line with anti-Vi serum within 48 hours, but no precipitation line with anti-O serum.

3.1.2 Chemical test

3.1.2.1 Total solid

It complies with the determination of total solid content (Appendix VII M).

3.1.2.2 Protein content

The protein content shall be less than 10 mg/g (Appendix VI B, method 2).

3.1.2.3 Nucleic acid content

The nucleic acid content shall be less than 20 mg/g. The extinction coefficient ($E_{1\text{cm}}^{1\%}$) of nucleic acid at 260 nm shall be 200 (Appendix II A).

3.1.2.4 O-acetyl content

The O-acetyl content shall be not less than 2 mmol/g (Appendix VI F).

3.1.2.5 Molecular size of polysaccharide

At least 50% of Vi polysaccharide shall be recovered in eluents before a distribution constant (K_D) of 0.25 is reached (Appendix VIII H).

3.1.3 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.2 Control tests on final bulk

3.2.1 Vi polysaccharide content

Add 0.6 g of agarose into 40 ml of 0.05 mol/L barbital buffer (pH 8.6), and swell it by heating. Add 1 ml of anti-Vi serum into it when cooled to 56°C, mix well and rapidly pour onto a clean glass plate. After coagulation of the gel, dig wells 3 mm in diameter at 1.5 cm away from the bottom edge. Add diluted typhoid Vi antigen standard solution (the concentrations are 100 µg, 50 µg, 25 µg, 12.5 µg and 6.25 µg/ml, respectively) and 5 µl of the sample (in duplicate) into the wells, respectively. Add 10 µl of bromophenol blue indicator into the well next to the edge. After loading sample, mount the glass plate on electrophoresis trough, and connect the end of sample loading with cathode of electrophoretic apparatus by using filter paper. Using 0.05 mol/L barbital buffer pH 8.6 as electrode buffer solution, perform electrophoresis under a constant voltage of 8 V/cm until the indicator migrates to the front. Immerse the glass plate in physiological saline for 1-2 hours, then cover it with clean filter paper and put into a incubator overnight for drying. Stain the gel with Coomassie brilliant blue staining solution until the rocket peak appears. Destain the gel in methanol-acetic acid destaining solution until the background of the gel is clear. Measure accurately the peak heights. A linear regression equation is obtained by regressing the log of concentration of standards with their peak heights. The concentration of the sample is obtained by inserting average value of its peak height into the regression equation.

3.2.2 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3 Control tests on final product

3.3.1 Identity test

See Section 3.1.1.

3.3.2 Inspection on final containers

The final product shall be a clear, colourless liquid free of foreign matters.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.5-7.5 (Appendix V A).

3.3.3.2 Preservative content

The content of phenol as a preservative shall be not more than 3.0 g/L (Appendix VI M).

3.3.3.3 Vi polysaccharide content

See Section 3.2.1 Vi polysaccharide content shall

be not less than 30 µg per single human dose.

3.3.4 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.5 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

3.3.6 Pyrogen test

It complies with the pyrogen test (Appendix VIII D). The injecting dose shall be 0.025 µg/kg of rabbit body weight.

4 Storage, shipping, and validity period

Store and ship at 2-8°C, protected from light. The validity period is 24 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Vi Polysaccharide Typhoid Vaccine

[Drug name]

Adopted name: Vi Polysaccharide Typhoid Vaccine

[Constituents and characters]

Vi polysaccharide typhoid vaccine is a purified Vi polysaccharide antigen extracted from the culture of *Salmonella typhi* and diluted with PBS. It is a clear, colourless liquid.

[Eligibles]

Military personnel, port and railway workers, sewage and rubbish disposal workers, caterers, medical and antiepidemic personnel, boat dwellers, and residents of endemic areas of typhoid fever.

[Function and usage]

The vaccine can induce humoral immune response in recipients following immunization. It is used to prevent typhoid fever.

[Specifications]

5 ml (10 human doses), 1 ml (2 human doses), or 0.5 ml (single human dose) per container. Each single human dose is 0.5 ml containing not less than 30 µg of typhoid Vi polysaccharide.

[Administration and dosage]

(1) The vaccine should be injected i.m. in the deltoid muscle of the lateral upper arm.

(2) A single injection of 0.5 ml shall be given to each recipient.

[Adverse reactions]

The adverse reactions are mild. Transient low fever occurs occasionally. Slight tenderness at the injection site may occur, which can be relieved spontaneously.

[Contraindications]

The vaccine should not be administered to the subjects with the following conditions:

(1) Fever, serious heart diseases, hypertension, hepatic and renal diseases, and active tuberculosis.

(2) Pregnancy, menstruation and lactation.

(3) A history of allergic reactions.

[Precautions]

- (1) Do not use the vaccine if any foreign matters or leakage of container is found.
- (2) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]**[Validity period]**

24 months.

[Standard for implementation]**[Product license number]****[Manufacturer]**

Name;
Address;
Zip code;
Tel;
Fax;
Web site;

Dysentery Vaccine(Live)of *S. flexneri* and *S. sonnei*, Oral

Dysentery vaccine of *Shigella flexneri* & *Shigella sonnei* is a preparation made by cultivation and harvest of FS strain expressing antigens of *S. flexneri* 2a and *S. sonnei* and lyophilization following addition of a stabilizer. It is used to prevent bacillary dysentery.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing**2.1 Bacterial seeds**

The bacterial seeds for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The bacterial strains used for production shall be genetically engineered FS strain expressing antigens of both *S. flexneri* 2a and *S. sonnei*.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lots

The subculture of the seed from master seed lot shall not exceed five passages.

2.1.4 Control tests on seed lots

2.1.4.1 Cultural characteristics and microscopic examination of stained smears

FS strain shall be Gram-negative bacilli and the smooth, translucent and colourless colonies shall grow on solid agar plate.

2.1.4.2 Biochemical reactions

FS strain shall ferment glucose and mannitol and slowly ferment galactose without production of gas. It shall not ferment lactose, maltose or sucrose. The strain shall be nonmotile (Appendix XIV).

2.1.4.3 Serological tests**(1) Slide agglutination test**

Slide agglutination test shall be performed on the culture after incubation at 35-37°C for 18-20 hours with anti-*S. flexneri* II; 3, 4 reference serum and anti-*S. sonnei* phase I reference serum, respectively. Agglutination shall be observed within 1 minute.

(2) Quantitative agglutination test

After incubation at 35-37°C for 18-20 hours, the culture shall be diluted with sterile PBS pH 7.2-7.4 to a suspension containing 1.0×10^9 bacilli/ml. Quantitative agglutination test shall be performed on the suspension with anti-*S. flexneri* II; 3, 4 reference serum and anti-*S. sonnei* phase I reference serum, respectively. The suspension shall be mixed thoroughly with the corresponding serum and allowed to stand at 35-37°C overnight. The highest dilution of serum, which gives an agglutination (+) observed visually, is regarded as agglutination titer. The agglutination titer shall be not less than half of the original titer of the reference serum.

2.1.4.4 Guinea pig corneo-conjunctival virulence test

Two Hartley guinea pigs each weighing 300-500 g are used as experimental models. One inoculating loop (3 mm in diameter, containing approximate 5×10^{10} viable bacilli) of the bacterial lawn incubated at 35-37°C for 18-20 hours is applied on their corneo-conjunctivae. The animals are observed for 7 days and no inflammatory reaction shall be seen.

2.1.4.5 Immunity test

Forty mice each weighing 14-16 g are divided into four groups with 10 mice each group. Two groups of animals are injected s.c. with 2.5×10^9 bacilli, and the other two with saline as a control. All the animals are immunized 3 times at an interval of 3 days. Fourteen days after the last immunization, both the immunized groups and the control groups shall be challenged with 50 LD₅₀ of *S. flexneri* 2a and *S. sonnei* strains and observed for 3 days. The protection rate shall be more than 70%.

2.1.4.6 Immunogenicity test

Inoculate the FS strain onto Hottinger slant or other appropriate media and incubate at 35-37°C for 18-20 hours. Collect and suspend the lawn into sterile PBS pH 7.2-7.4 and dilute it to a certain concentration. Immunize three rabbits each weighing about 2 kg 4 times through auricular veins with 0.5 ml of the bacterial suspension at an

interval of 5-7 days. Four injecting doses contain 2.5×10^8 , 5.0×10^8 , 1.0×10^9 and 2.0×10^9 bacilli, respectively. Collect blood sample for a quantitative agglutination test to determine serum antibody titer 10-14 days after the last injection. The serum agglutination titers shall be not less than 1 : 1280 for *S. flexneri* 2a and not less than 1 : 320 for *S. sonnei*. The FS strain is judged as qualified if the agglutination titers of serum in two-thirds of rabbits reach the above levels.

2.1.4.7 Plasmid DNA test

The plasmid DNA is extracted from FS culture by Kado-Liu method. A typical plasmid map of the strain shall be shown by 0.8% agarose gel electrophoresis and there shall be two large plasmid bands with molecular weights of 49 MD and 74 MD respectively and two small plasmid bands.

2.1.5 Storage of bacterial seeds

The bacterial seeds shall be lyophilized and stored at 2-8°C.

2.2 Bulk

2.2.1 Working seed lots for production

Working seed lots can be used within 2 years after satisfactory control tests. They shall be subject to overall test before each production and only those qualified in the test can be used for production.

The freeze-dried seeds from working seed lot shall be reconstituted with sterile PBS pH 7.2-7.4 or Hottinger broth and inoculated onto Hottinger slant or into Hottinger liquid media. The culture shall be incubated at 35-37°C for 18-20 hours. The second and third passages of strains shall be inoculated into Hottinger liquid medium and incubated at 35-37°C for 6-8 hours to prepare the working seed for production.

2.2.2 Production medium

The Hottinger liquid medium or other appropriate media shall be used.

2.2.3 Inoculation and cultivation of bacterial seed

The bacterial seed qualified in bacterial purity test shall be inoculated into medium in a fermentor to an initial concentration of 4×10^8 bacilli/ml or more and cultivated at 35-37°C for 8-11 hours. The samples shall be taken for microscopic examination during cultivation. If any contaminating microorganisms are found, the culture shall be discarded.

2.2.4 Harvest

Harvest the bacteria during the late logarithmic phase and suspend in a sterile stabilizer after centrifugation.

2.2.5 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

The bacteria suspended in a stabilizer shall be diluted to a concentration of 1.0×10^{11} bacilli/ml

with PBS containing 5% of sucrose and 0.5% of gelatin. The final bulk can be prepared from either single batch or pooled batches of bulk.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The final product shall be lyophilized immediately after filling. The temperature of product shall be not higher than 30°C during lyophilization. After lyophilization, the container shall be sealed immediately under vacuum or after filling with nitrogen.

2.4.3 Specifications

1 ml of reconstituted vaccine per container containing 1.0×10^{11} bacilli, with the number of culturable particles not less than 2.0×10^{10} .

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Bacterial purity test

The sample shall be inoculated onto Hottinger agar slant and incubated at 35-37°C for 48 hours. No growth of contaminating microorganisms shall be observed.

3.1.2 Bacterial content

The bacterial content shall be determined against the National Standard of Bacterial Opacity.

3.2 Control tests on final bulk

3.2.1 Bacterial purity test

See Section 3.1.1.

3.2.2 Test for percentage of culturable particles

The bacterial suspension shall be diluted to a concentration of 1.0×10^{11} bacilli/ml according to the National Standard of Bacterial Opacity. The number of culturable particles shall be determined by plate count method and its percentage shall be not less than 25%.

3.3 Control tests on final product

Other than the determination of moisture content, the product shall be reconstituted with sterile PBS (pH 7.2-7.4) as stated on the label and subject to the following tests.

3.3.1 Identity test

The product shall be streaked onto Hottinger agar slant and incubated at 35-37°C for 18-20 hours. The lawn shall be collected for slide agglutination tests with anti-*S. flexneri* II; 3, 4 sera and anti-*S. sonnei* phase I serum. Distinct agglutination

shall be observed.

3.3.2 Inspection on final containers

The product looks like a milky-white or slightly yellow crisp cake and shall be reconstituted with sterile PBS within 1 minute.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Bacterial purity test

See Section 3.1.1.

3.3.5 Test for number of culturable particles

The number of culturable particles shall be determined by plate count method. The number of culturable particles of final product shall be not less than 2.0×10^{10} particles/ml.

3.3.6 Immunogenicity test

One container of the final product shall be inoculated onto Hottinger slant after reconstitution. The culture shall be incubated at 35-37°C for 18-20 hours. The lawn shall be collected and suspended in sterile PBS pH 7.2-7.4 (See Section 2.1.4.6). One of every ten batches shall be sampled for the test.

3.3.7 Guinea pig corneo-conjunctival virulence test

See Section 2.1.4.4.

3.3.8 Safety test

Weigh five mice weighing 18-22 g, respectively, and feed each with 1.0×10^{10} bacteria. Observe the animals for 7 days, and they shall survive with weight gain. The test may be repeated in ten mice if the results fail to meet the above-mentioned requirements.

3.4 Control tests on diluent

Each bag shall contain 0.1-0.2 g of sodium bicarbonate, 0.05-0.1 g of sodium ascorbate and 0.05-0.1 g of molasses, which are dissolved in 50 ml of water for injection.

3.4.1 pH

The pH shall be 7.5-8.5 (Appendix V A).

3.4.2 Microbial limit test

It complies with the test for microbial limit (Appendix VIII G).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 12 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Dysentery Vaccine (Live) of *S. flexneri* & *S. sonnei*, Oral

[Drug name]

Adopted name: Dysentery Vaccine (Live) of *S. flexneri* and *S. sonnei*, Oral

[Constituents and characters]

The product is made by cultivation and harvest of FS strain expressing antigens of *S. flexneri* 2a and *S. sonnei* and lyophilization after addition of a

stabilizer. It looks like a milky-white or slightly yellow crisp cake.

[Eligibles]

All age groups are suitable for immunization.

[Function and use]

The vaccine can induce immune response in recipients following oral administration. It is used to prevent bacillary dysentery.

[Specifications]

1 ml per container containing 1.0×10^{11} bacilli with the number of culturable particles not less than 2.0×10^{10} .

[Administration and dosage]

Three doses should be given orally at an interval of 5-7 days. For adult, one container of the vaccine should be administered for the first dose and two containers for the second and third doses, separately; for children aged 6-13 years, half of the adult dosage should be given; for children under 5 years, one-third of the adult dosage should be given.

Dissolve one bag of powder (0.1-0.2 g of sodium bicarbonate, 0.05-0.1 g of sodium ascorbate and 0.05-0.1 g of molasses) in 50 ml of cool boiled water to prepare diluent solution. Open the vaccine container, add a small amount of the solution into the container with an accompanying pipette. Transfer the reconstituted product in the diluent solution and mix well for oral administration.

[Adverse reactions]

Occasionally slight reactions such as nausea and abdominal discomfort may occur.

[Contraindications]

The vaccine should not be administered to the subjects with the following conditions:

- (1) Immunodeficiency or immunologic inadequacy.
- (2) Gastrointestinal, cardiac, hepatic and renal diseases.
- (3) Acute infectious diseases or fever.

[Precautions]

- (1) Injection is strictly contraindicated!
- (2) Orally administer the vaccine before or 2 hours after a meal.
- (3) Administer the vaccine immediately following opening the container.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

12 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:
Address:

Zip code:
Tel:
Fax:
Web site:

Group A Meningococcal Polysaccharide Vaccine

Group A meningococcal polysaccharide vaccine is a purified capsular polysaccharide antigen extracted from the culture of *Neisseria meningitidis* group A and lyophilized after addition of an appropriate stabilizer. It is used to prevent epidemic cerebrospinal meningitis caused by *Neisseria meningitidis* group A.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The bacterial strain used for production shall be *Neisseria meningitidis* group A strain CMCC 292021 (A4).

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lot

The subculture of the seed from master seed lot shall not exceed five passages; the subculture of the seed from working seed lot inoculated into a fermentor shall not exceed five passages.

2.1.4 Control tests on seed lots

2.1.4.1 Cultural characteristics and microscopic examination of stained smears

When the bacterial seed is inoculated onto a common agar medium containing 10% sheep blood, meningococci shall not grow at 25°C. The bacteria shall be incubated at 35-37°C in Carbon dioxide incubator for 16-20 hours, and smooth, humid and greyish-white colonies shall grow. The lawns shall be removed easily and can be made into a homogeneous suspension in physiological saline. The cultures shall be Gram-negative diplococci or single cocci by microscopic examination of stained smears.

2.1.4.2 Biochemical reactions

The cultures shall ferment glucose and maltose

with production of acid but not gas. They shall not ferment lactase, mannitol, fructose or sucrose (Appendix XIV).

2.1.4.3 Serum agglutination test

After incubation at 35-37°C for 16-20 hours, the lawn shall be inactivated by suspending in the physiological saline containing 0.5% formalin or by heating at 56°C for 30 minutes. Dilute the suspension to a concentration of 1.0×10^9 - 2.0×10^9 bacteria/ml. Perform a quantitative agglutination test with reference group A serum. The mixture shall be kept at 35-37°C overnight and then at room temperature for 2 hours the day after and observed visually. The highest dilution of serum which gives a clear agglutination (+) is regarded as the agglutination titer. The agglutination titer shall be not less than half of the original titer of the serum.

2.1.5 Storage of seed lot

Primary seed lot and master seed lot shall be stored lyophilized at 2-8°C.

2.2 Bulk

2.2.1 Working seed lots for production

After all characteristics are examined as qualified, the bacterial seed derived from working seed lot shall be inoculated onto a modified semisynthetic medium or other appropriate media to prepare a quantity of working seeds for production.

2.2.2 Production medium

The modified semisynthetic medium or other appropriate media shall be used for production.

The media shall be free from ingredients that may form precipitate with hexadecyltrimethylammonium bromide.

2.2.3 Cultivation

Inoculate the bacterial seed into liquid medium in a fermentor. Take samples during cultivation and test for bacterial purity by microscopic examination of Gram-stained smears. If any contaminating microorganisms are found, the cultures shall be discarded.

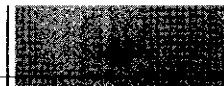
2.2.4 Harvest and killing

Terminate cultivation at the late logarithmic or early stationary phase, and take samples to test for bacterial content and purity. After qualified in the tests, the harvested bacteria shall be killed by addition of formalin or by heating. It is proper to ensure complete killing of bacteria without damage to its polysaccharide antigen.

2.2.5 Purification

2.2.5.1 Removal of nucleic acid

After killing of the bacteria, centrifuge the cultures (a single harvest or pooled harvests), and collect the supernatant. To the supernatant, add hexadecyltrimethylammonium bromide and mix well to form a precipitate. To the precipitate, add a quantity of calcium chloride solution to a final concentration of 1 mol/L, and dissociate the



polysaccharide from hexadecyltrimethylammonium bromide. Add ethanol to a final concentration of 25% and allow to stand at 2-8°C for 1-3 hours or overnight. Collect the clear supernatant by centrifugation.

2.2.5.2 Precipitation of polysaccharide

To the above-mentioned supernatant add cold ethanol to a final concentration of 80 %, mix well to precipitate the polysaccharide, and collect the precipitate following centrifugation. Wash the precipitate with absolute ethanol and acetone for 2 times or more, respectively, to prepare crude polysaccharide. Store the crude polysaccharide at or below -20°C for purification.

2.2.5.3 Purification of polysaccharide

Dissolve the crude polysaccharide in 1/10 saturated neutral sodium acetate solution to a concentration of 10-20 mg/ml, then extract several times with twice its volume of cold phenol (dissolve 100 g of crystalline phenol in 40 ml of 1/10 saturated sodium acetate). Collect the supernatant by centrifugation, and dialyse against 0.1 mol/L calcium chloride solution or other appropriate solutions. To the supernatant, add ethanol to a final concentration of 75%-80%. Collect the precipitated polysaccharide by centrifugation, and wash it with absolute ethanol and acetone for 2 times or more, respectively. After drying, dissolve the precipitate in sterile water for injection to prepare the bulk purified polysaccharide. Purification process shall be carried out at or below 15°C.

2.2.6 Control tests on bulk

Take samples from the bulk purified polysaccharide after sterilization by filtration. See Section 3.1.

2.2.7 Storage and storage period

The bulk shall be stored at or below -20°C. The storage period is 60 months starting from the date when crude polysaccharide is produced.

2.3 Final bulk

2.3.1 Formulation

The final bulk shall be prepared from either a single batch or pooled batches of bulk polysaccharide. Add sterile pyrogen-free lactose and dilute with sterile water for injection. Each single human dose contains 30 µg of polysaccharide and 2.5-3.0 mg of lactose.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The temperature of product

during lyophilization shall be not higher than 30°C. The container shall be sealed under vacuum or after filling with nitrogen.

2.4.3 Specifications

150 µg or 300 µg per container. Polysaccharide content shall be not less than 30 µg per single human dose.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control Tests

3.1 Control tests on bulk

3.1.1 Identity test

Carry out the identity test by the method of double immunodiffusion (Appendix VIII C). The sample shall form an apparent precipitation line with the antibody to *Neisseria meningitidis* group A.

3.1.2 Chemical tests

3.1.2.1 Total solid

It complies with the determination of total solid content (Appendix VIII M).

3.1.2.2 Protein content

The protein content shall be less than 10 mg/g (Appendix VI B, method 2).

3.1.2.3 Nucleic acid content

The nucleic acid content shall be less than 10 mg/g (Appendix II A). The extinction coefficient ($E_{1\text{cm}}^{1\%}$) of nucleic acid at 260 nm shall be 200.

3.1.2.4 O-acetyl content

The O-acetyl content shall be not less than 2 mmol/g (Appendix VI F).

3.1.2.5 Phosphorus content

The phosphorus content shall be not less than 80 mg/g (Appendix VII A).

3.1.2.6 Molecular size of polysaccharide

The distribution constant (K_D) of the polysaccharide molecule shall be not more than 0.40. At least 65% of the polysaccharide shall be recovered in eluents before K_D value of 0.5 is reached (Appendix VIII G).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Test for bacterial endotoxin

Endotoxin content of polysaccharide shall be not more than 100 EU/µg (Appendix XII E, gel limit test). Alternatively, pyrogen test can be carried out (Appendix XII D). The injecting dose shall be 0.025 µg of polysaccharide/kg of rabbit body weight.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content, the product shall be reconstituted with sterile PBS as stated on the label and subject to the following tests.

3.3.1 Identity test

Carry out the identity test by the method of double immunodiffusion (Appendix VIII C). The polysaccharide product shall form an apparent precipitation line with antibody to *Neisseria meningitidis* group A.

3.3.2 Inspection on final containers

The freeze-dried product looks like a white crisp cake. After reconstitution with PBS as stated on the label, it shall turn into a clear liquid free of foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 Polysaccharide content

The polysaccharide content shall be not less than 30 µg per single human dose. According to the formula specified by WHO Requirements (the ratio of polysaccharide content to phosphorus content is 1000 : 75), polysaccharide content shall be calculated following determination of phosphorus content which shall be not less than 2.25 µg per dose (Appendix VII A).

3.3.3.3 Molecular size of polysaccharide

At least one of every five batches shall be sampled for determination of molecular size of polysaccharide. The distribution constant (K_D) shall be not more than 0.40. At least 65% of the polysaccharide shall be recovered from the column before K_D value of 0.5 is reached (Appendix VIII G).

3.3.4 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.5 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F). The injecting dose for each guinea pig shall be 500 µg/ml and for each mouse shall be 100 µg/0.5 ml.

3.3.6 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose shall be 0.025 µg of polysaccharide/kg of rabbit body weight.

3.4 Control tests on diluent

The diluent is sterile pyrogen-free PBS.

3.4.1 pH

The pH shall be 6.8-7.2 (Appendix V A).

3.4.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.4.3 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.4.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose shall be 1 ml/kg of rabbit body weight.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 24 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Group A Meningococcal Polysaccharide Vaccine

[Drug name]

Adopted name: Group A Meningococcal Polysaccharide Vaccine

[Constituents and characters]

The vaccine is a purified capsular polysaccharide antigen extracted from the culture of *Neisseria meningitidis* group A and lyophilized after the addition of an appropriate stabilizer. The final product looks like a white crisp cake. After reconstitution it shall turn into a clear liquid.

[Eligibles]

Children at the age of 6 months to 15 years.

[Function and use]

The vaccine can induce humoral immune response in recipients following immunization. It is used to prevent epidemic cerebrospinal meningitis caused by *Neisseria meningitidis* group A.

[Specifications]

150 µg or 300 µg of polysaccharide per container. Each single human dose contains 30 µg of polysaccharide.

[Administration and dosage]

(1) Open the container and reconstitute vaccine with the accompanying diluent as stated on the label. Shake and inject immediately.

(2) The vaccine should be injected s.c. at deltoid insertion area of the lateral upper arm at a dosage of not less than 30 µg of polysaccharide (in 0.2 ml or 0.5 ml).

(3) The primary immunization consisting of two injections for children shall begin at 6 months of age, at an interval of 3 months. One booster shall be given to the children at 3 years of age or older before the epidemic season.

Revaccinations may be given every 3 years, if needed. The vaccine may be administered to other age groups for emergent vaccination during epidemic.

[Adverse reactions]

The adverse reactions are mild. Transient low fever occurs occasionally. Slight tenderness at the injection site may occur, which can be relieved spontaneously.



[Contraindications]

The vaccine should not be administered to the subjects with the following conditions:

- (1) Epilepsy, convulsion, brain diseases, and a history of allergic reactions.
- (2) Renal diseases, heart diseases, and active tuberculosis.
- (3) Acute infectious diseases and fever.

[Precautions]

- (1) Do not use the vaccine if loose stopper, leakage of container or foreign matters after reconstitution are found.
- (2) Once the product in a container is reconstituted, it shall be used up at one time according to the specified dosage. The remaining vaccine shall be discarded.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;
Address;
Zip code;
Tel;
Fax;
Web site;

Leptospira Vaccine

Leptospira vaccine is a monovalent or polyvalent vaccine made by cultivation and inactivation of the cultures of different serovars of leptospira interrogans, which are mainly prevalent in various areas. It is used to prevent leptospirosis.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Leptospiral strains

The leptospiral strains for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds for Production and Quality Control of Biologics.

2.1.1 Name and origin of strains

The main strains of leptospira for production shall be as follows:

Serogroup	Serovar	Strain	Virulence
Icterohaemorrhagiae	Lai	Lai,017, Jiang 4,70091	High
Canicola	Canicola	611,Gui 44	Low
Pyrogens	Pyrogens	[4],HBS 5	High
Autumnalis	Autumnalis	Lin 4	High
Australis	Australis	Wo 34,115,620	Low
Pomona	Pomona	Luo,109	Low
Grippotyphosa	Linhai	Lin 6	High
Hebdomadis	Hebdomadis	401,Guang 229,245	Low

2.1.2 Establishment of leptospiral strains

It complies with the Requirements for Bacterial and Viral Strains/Seeds for Production and Quality Control of Biologics.

2.1.3 Control tests on leptospiral strains

The leptospiral strains shall be passaged by inoculation in guinea pigs, each weighing 120-220 g. The heart blood shall be drawn or liver tissue shall be taken 2-3 days after inoculation or on the brink of death to inoculate onto production medium or other appropriate media, and control tests shall be performed on leptospiral cultures of 4th passage or more.

2.1.3.1 Morphological and cultural characteristics

The leptospiral strain shall be inoculated into production medium with an inoculum of less than 5% and incubated at 28-32°C for 5-10 days. The cultures shall contain more than 100 organisms/400× microscopic field. The cultures shall be transparent with opalescence, and slightly turbid on shaking. Leptospira appear as mobile rods with regular shapes and both ends curved.

2.1.3.2 Serum agglutination test

A quantitative agglutination test shall be performed with reference serum on live, 3-10-day-old cultures containing 50-100 mobile organisms/400× microscopic field, without self-agglutination. The agglutination titer shall be not less than half of the titer of the original serum. The number of leptospira decreasing by 50% shall be judged as the endpoint of titer. Serological typing shall be performed on new leptospiral strains by agglutinin cross absorption test.

2.1.3.3 Virulence test

The test shall be performed by subcutaneous injection of 2 ml of live 5-10-day-old cultures to be tested containing 50-100 organisms/400× microscopic field into each of six guinea pigs weighing 180-220 g of two groups, respectively. In one group, the blood shall be drawn from heart of guinea pigs 48 hours after injection. Inoculate the blood (1% in proportion to the medium) into two tubes of production medium or other appropriate media, incubate for 14 days, and examine by microscopy. If the cultures are positive (growth of leptospira), the strain shall be judged as low virulent strain. In the other group, the guinea pigs are observed for 10 days after injection. It shall be judged as highly

virulent strain if at least two guinea pigs die of leptospirosis.

For known low or highly virulent strains stored through passages, the virulence test shall also be performed by the above corresponding method, respectively. It is judged as qualified if the strain meets the requirements for low or highly virulent strain.

2.1.3.4 Immunity test

The 5-10-day-old leptospiral cultures containing 70-100 organisms/400× microscopic field is inactivated by heating at 56-58°C for 1 hour or adding 3.0 g/L of phenol, and diluted 3-fold with physiological saline. Each of three guinea pigs weighing 120-220 g (the three guinea-pigs in control group should be fed at the same time) shall be given two injections of the diluted cultures by subcutaneous route at an interval of 5 days. The dosage is 0.5 ml for the first injection, and 1 ml for the second injection. The guinea pigs shall be challenged s.c. 10-12 days after the last injection with 2 ml of 5-10-day-old cultures of the same or homotypic strain containing 50-100 organisms/400× microscopic field.

Highly virulent strain: The guinea pigs shall be observed for 10 days after challenge. The animals in immunized group shall survive, and have normal appearance, appetite, activity, and weight gain, and no pilo-erection or jaundice. If at least two guinea pigs in control group die of leptospirosis, it shall be judged as qualified.

Low virulent strain: Draw blood from the heart of guinea pigs 24 hours after challenge, inoculate one to two drops of blood (about 1% of inocula) into each of two tubes of medium containing 5%-8% rabbit serum, and incubate for 14 days. The test shall be judged as qualified if more than two thirds of heart blood cultures in immunized group are negative, and all cultures in control group are positive.

2.1.3.5 Antigenicity test

The 5-10-day-old leptospiral cultures containing 70-100 organisms/400× microscopic field are inactivated by heating at 56°C for 1 hour. Each of three healthy rabbits weighing 2.0-2.5 kg shall be given three injections of 1 ml, 2 ml and 5 ml of the leptospiral cultures by intravenous routes respectively, at intervals of 5 days. An agglutination test shall be performed with the rabbit serum on the cultures of the same strain 10-15 days after the last injection. The test shall be judged as qualified if the serum titers of at least two rabbits reach 1 : 10000 or more.

2.1.4 Passage and storage of leptospiral strains

2.1.4.1 Passage of leptospiral strains

To preserve the virulence and purity of the leptospiral strains, after 3-6 passages, the strain shall be passaged in guinea pigs each weighing 120-220 g, and determined for serological and biological characteristics. The leptospiral strain

can be used for passage if the determination is qualified.

2.1.4.2 Storage of leptospiral strains

Leptospiral strains shall be stored in medium containing rabbit serum or other appropriate media at 18-22°C in dark place. The strains shall be passaged at regular intervals or stored in liquid nitrogen.

2.2 Bulk

One strain of each serovar of leptospira shall be used for vaccine production.

2.2.1 Working strains for production

Each guinea pig weighing 120-220 g shall be injected s.c. with 2 ml of well-grown 5-10-day-old leptospiral cultures. Heart blood shall be drawn and liver tissue shall be taken 2-3 days after injection or on the brink of death. Less than 1% of inoculation amount of blood or liver tissue shall be inoculated into production medium or other appropriate media, incubated at 28-32°C for 7-18 days, and up to 30 days for individual strain which grows with difficulty. After qualified in the test for bacterial purity and serological characteristics, the strains shall be passaged in production medium or other appropriate media for at least four passages. Only the well-grown, mobile, and pure cultures can be used for inoculation in large amounts.

The strain stored in liquid nitrogen shall be used for production immediately after reconstitution.

2.2.2 Production medium

Synthetic or semisynthetic medium shall be used for production.

2.2.3 Cultivation

Leptospiral strains shall be cultivated by aeration in 10 L bottles or in fermentor at 28-32°C for 4-14 days, and the number of leptospira shall reach 300/400× microscopic field or more. Samples of the cultures shall be taken for bacterial purity test and microscopic examination, and no contaminating microorganisms shall be found. The cultures shall be concentrated by appropriate methods.

2.2.4 Killing

The cultures shall be inactivated with phenol (its content shall be not more than 3.0 g/L) or other appropriate bactericides. The cultures shall be placed for at least 30 minutes. Samples of cultures shall be taken for microscopic examination for effective inactivation. Cultures in fermentor can be pooled and then inactivated. Each bottle shall be retested for sterility before pooling if leptospiral suspension is stored for more than 6 months.

2.2.5 Control tests on bulk

See Section 3.1.

2.2.6 Storage of bulk

The bulk shall be stored at 2-8°C.

2.3 Final bulk

2.3.1 After inactivation the leptospiral cultures of various serovars shall be mixed into one batch according to predetermined proportion.

2.3.2 Vaccine shall be made from the leptospiral cultures consisting of the main epidemic serovars. In the vaccine of not more than pentavalent, each serovar shall contain not less than 1.5×10^8 leptospira/ml; in the vaccine of not less than hexavalent, each serovar shall contain not less than 1.0×10^8 leptospira/ml. The content of leptospira for various serovars shall not deviate from the required content by more than 10%, and the total content of leptospira shall not exceed 1.25×10^9 leptospira/ml.

2.3.3 Sodium chloride shall be added to the vaccine to a final concentration of 7.5-9.5 g/L.

2.3.4 Control tests on final bulk
See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

5 ml per container.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Bacterial content

The content of leptospira shall be determined by the method of microscopic counting.

3.1.2 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.1.3 Sterility test

It complies with the test for sterility (Appendix VII A).

The cultures from each bottle shall be tested for sterility if incubated in large bottles.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix VII A).

If the cultures in fermentor are transferred into large bottles, samples shall be taken at the beginning, middle, and late transferring stages for sterility test.

3.3 Control tests on final products

3.3.1 Identity test

A seroagglutination test shall be performed on the product with antisera to the leptospiral antigens of

the serovars included in the vaccine, and specific agglutination shall occur.

3.3.2 Inspection on final containers

The product is a liquid with slight opalescence, free of abnormal odour, foreign matters and clumps not dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 (Appendix V A).

3.3.3.2 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.3 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.3.4 Potency test

The potency tests shall be performed based on the serovars of leptospira included in the vaccine. The vaccine shall be diluted with physiological saline to a concentration of 5×10^7 leptospira/ml. See Section 2.1.3.4.

3.3.5 Sterility test

It complies with the test for sterility (Appendix VII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 18 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Leptospira Vaccine

[Drug name]

Adopted name: Leptospira Vaccine

[Constituents and characters]

The product is a monovalent or polyvalent vaccine made by cultivation and inactivation of the cultures of different serovars of leptospira interrogans, which are mainly prevalent in various areas. It is a liquid with slight opalescence, and contains phenol as a preservative.

[Eligibles]

People at 7-60 years of age in epidemic areas.

[Function and use]

The vaccine can induce immune response in the recipients following immunization. It is used to prevent leptospirosis.

[Specifications]

5 ml per container.

[Administration and dosage]

(1) The vaccine should be injected s. c. at deltoid insertion area of the lateral upper arm.

(2) Adults: first injection of 0.5 ml and second

injection of 1.0 ml shall be given at an interval of 7-10 days.

Children at 7-13 years of age: the dosage shall be half of the adult dosage.

Children under 7 years of age: a proper dosage, but not more than one fourth of adult dosage shall be injected if necessary.

Vaccination shall be completed before epidemic seasons.

[Adverse reactions]

Systemic and local reactions are generally mild. Fever as well as local pain, erythema and swelling at the injection site may occur occasionally, which can be relieved spontaneously.

[Contraindications]

The vaccine shall not be administered to the subjects with the following conditions:

- (1) Fever, acute infectious diseases, serious heart diseases, hypertension, hepatic and renal diseases, neuropathy and psychosis.
- (2) Pregnancy, lactation and menstruation.
- (3) A history of allergic reactions.

[Precautions]

(1) Do not use the vaccine if any leakage of container, foreign matters or clumps not dispersed on shaking are found, or the product has been frozen.

(2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(3) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Plague Vaccine (Live) for Percutaneous Scarification

Live plague vaccine for percutaneous scarification is a preparation made by the cultivation and harvest of a live attenuated strain of *Yersinia pestis* and lyophilization following addition of a stabilizer. It is used to prevent plague.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The attenuated EV strain of *Yersinia pestis* shall be used for vaccine production.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passages of seed lots

The subculture of freeze-dried working seed used for production shall be the second or third passage. The fourth passage of the bacterial seed shall not be used for production.

2.1.4 Control tests on seed lots

2.1.4.1 Cultural characteristics and microscopic examination of stained smears

Inoculate the seed onto agar plates and incubate at 35-37°C for 44-48 hours. The colonies shall be rough type. The bacilli cultivated in broth medium shall form a thin pellicle on the surface and a precipitate at the bottom of the broth, while the broth itself shall remain clear. The cultures shall be Gram-negative rods by microscopic examination of stained smears.

2.1.4.2 Biochemical reactions

The cultures shall ferment glucose with the production of acid but not gas, and turn the litmus milk into slight red. No acid or gas shall be produced in glycerin medium (Appendix XIV).

2.1.4.3 Phage bacteriolytic test

Inoculate and spread the bacteria of EV strain onto agar plate and add one drop of plague phage at a dilution of not less than 10^{-6} . Incubate the plate at 28°C for 44-48 hours. There shall be no growth of the bacteria at the site where the plague phage was dropped.

2.1.4.4 Tests for residual virulence

Inject s. c. each of three guinea pigs weighing 300-400 g with a suspension of 1.2×10^{10} bacilli (the cultures cultivated at 28-30°C for 44-48 hours). One guinea pig shall be autopsied on the 6th day and the other two on the 21st day after injection. Examine visually the injection site, spleen, liver and lung, and incubate the samples taken from spleen, liver, lung and heart blood. No growth of the bacilli shall be found in the cultures of heart blood and lung tissue. Congestion and the infiltration that may turn into abscess may occur at

the injection site, and papule-like nodules may occur in the liver and spleen, but no specific plague lesions shall be found in the lungs. If the specific plague lesions appear obviously in the lungs, the test shall be repeated with the same number of guinea pigs. If the specific plague lesions are still found, the seed shall be discarded.

2.1.4.5 Immunity test

Immunize s.c. each of ten guinea pigs weighing 200-250 g with a suspension of 7.0×10^9 bacilli (the cultures cultivated at 28-30°C for 44-48 hours). Challenge s.c. each of the immunized guinea pigs with 200 MLD of *Yersinia pestis* 20-25 days after immunization. At the same time, three groups each consisting of three guinea pigs shall be used as controls. The guinea pigs in the three control groups shall be injected each with 0.5 MLD, 1 MLD and 2 MLD of *Yersinia pestis*, respectively. Both the immunized and the control groups shall be observed for at least 25 days. The immunity test is qualified if at least eight animals in the immunized group survive, while in the control groups, the animals injected with 0.5 MLD of bacilli die partially, and those with 1 MLD or 2 MLD die totally.

2.1.5 Storage of seed lots

The seed lots shall be stored lyophilized at 2-8°C, and protected from light.

2.2 Bulk

2.2.1 Working seed lots for production

2.2.1.1 The first passage of bacterial seed

Inoculate the bacterial seed from working seed lot on Hottinger agar medium and incubate at 28-30°C for 44-48 hours to prepare the first passage of seed. It shall be stored at 2-8°C and can be used within 15 days.

2.2.1.2 The second passage of bacterial seed

Wash down the bacterial lawn of the first passage with physiological saline and inoculate into seed bottles. Incubate at 28-30°C for 44-48 hours to obtain the second passage from which a quantity of working seeds for production shall be prepared.

2.2.1.3 The third passage of bacterial seed

If the second passage of bacterial seed is not enough for production, wash down the bacterial lawn of the second passage with physiological saline and inoculate into seed bottles. Incubate at 28-30°C for 44-48 hours to obtain the third passage from which a quantity of working seeds for production shall be prepared.

2.2.2 Production medium

The Hottinger agar medium pH 6.8-7.2 or other approved appropriate media shall be used for production.

2.2.3 Inoculation and cultivation

If no contaminating microorganisms are found in the second or third passage of bacterial seed by visual inspection, wash down the bacterial lawn

with a quantity of physiological saline to make a homogenous suspension. Inoculate the suspension into culture bottles and cultivate at 28-30°C for 44-48 hours. The culture bottles shall be inspected one by one, and the contaminated ones shall be discarded.

2.2.4 Harvest and pooling

Harvest the cultures by washing down the bacterial lawn with a stabilizer containing sucrose, gelatin, thiourea, sodium glutamate and urea (remove the condensed water before washing), or by scraping the bacterial lawn into the stabilizer. The harvests can be pooled to prepare a bulk if the test for the absence of contaminating microorganisms proved qualified.

2.2.5 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

Dilute the bulk with a stabilizer to a concentration of 7×10^8 - 9×10^8 bacilli per single human dose according to the bacterial content determined in Section 3.1.2.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The products shall be lyophilized immediately after filling. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.4.3 Specifications

8×10^9 bacilli per container for ten human doses, or 1.6×10^{10} bacilli per container for twenty human doses. Each single human dose shall contain more than 3.6×10^8 live bacilli.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Test for the absence of contaminating microorganisms

It complies with the bacterial purity test (Appendix VII A). No contaminating microorganisms shall be found in the cultures by microscopic examination of stained smears. The bacteria shall meet the characteristics of *Yersinia pestis* EV strain.

3.1.2 Bacterial content

The bacterial content shall be determined against the National Standard of Bacterial Opacity.

3.2 Control tests on final bulk

It complies with the bacterial purity test (Appendix VII A). No contaminating microorganisms shall be found in the cultures by microscopic examination of stained smears. The bacteria shall meet the characteristics of *Yersinia pestis* EV strain.

3.3 Control tests on final product

Other than the determination of moisture content, the product shall be reconstituted with sodium chloride injection as stated on the label and subject to the following tests.

3.3.1 Identity test

See Section 2.1.4.3.

3.3.2 Inspection on final containers

The freeze-dried product looks like a white or yellowish crisp cake. The product shall be reconstituted into a homogenous suspension within half a minute after addition of physiological saline as stated on the label.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Test for the absence of contaminating microorganisms

It complies with the bacterial purity test (Appendix VII A). No contaminating microorganisms shall be found in the cultures by microscopic examination of stained smears. The bacteria shall meet the characteristics of *Yersinia pestis* EV strain.

3.3.5 Inspection for morphology and colony of bacilli

The bacilli shall be Gram-negative rods by microscopic examination of stained smears and the colony shall be typically rough.

3.3.6 Bacterial content

The bacterial content shall be determined against the National Standard of Bacterial Opacity. Each human dose of the vaccine shall contain 7×10^8 - 9×10^8 bacilli.

3.3.7 Test for the number of culturable particles

Reconstitute the product in three containers into a homogeneous bacterial suspension with physiological saline. The suspension shall be diluted to a concentration of 1.0×10^3 bacilli/ml. Inoculate 0.1 ml of diluted suspension onto each of five plates and spread evenly, and incubate at 28-30°C for 2-3 days. Each human dose of the vaccine shall contain more than 3.6×10^8 bacilli.

3.3.8 Potency test

The first batch of every five batches shall be sampled for the potency test. Immunize s.c. each of ten guinea pigs weighing 250-300 g with the product containing 5.0×10^7 bacilli. Challenge s.c. each of the guinea pigs with 200 MLD of *Yersinia pestis* 20-25 days after immunization. At the same time, three groups each consisting of three guinea pigs shall be used as controls. The

guinea pigs in the three control groups shall be injected s.c. each with 0.5 MLD, 1 MLD and 2 MLD of *Yersinia pestis*, respectively. The animals in each group shall be observed for 25 days. The product is qualified in potency test if at least eight animals in the immunized group survive, while in the control groups, the animals injected with 0.5 MLD die partially, and those with 1 MLD or 2 MLD die totally.

3.3.9 Specific toxicity test

Each of two guinea pigs weighing 250-350 g shall be injected s.c. with the product at a dosage of 1.2×10^{10} bacilli. Weigh the animals on the 6th day after the injection, and autopsy one of them. The loss of body weight shall be not more than 20%. The other guinea pig shall be autopsied on the 21st day after injection. The tests shall be carried out according to the methods given in Section 2.1.4.4.

3.3.10 Diluent

The diluent shall be sodium chloride injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 12 months starting from the date when the test for the number of culturable particles proved qualified.

5 Package inserts

Directions for Use of Plague Vaccine (Live) for Percutaneous Scarification

[Drug name]

Adopted name: Plague Vaccine (Live) for Percutaneous Scarification

[Constituents and characters]

Plague vaccine for percutaneous scarification is a preparation made by cultivation and harvest of a live attenuated strain of *Yersinia pestis* and lyophilization following addition of a stabilizer. It looks like a white or yellowish crisp cake and shall be a homogenous suspension after reconstitution.

[Eligibles]

Inhabitants living in the endemic area and those who intend to enter the area from non-endemic area.

[Function and use]

The vaccine can induce immune response in the recipients following immunization. It is used to prevent plague.

[Specifications]

8.0×10^9 bacilli per container for ten human doses, or 1.6×10^{10} bacilli per container for twenty human doses. Each single human dose shall contain more than 3.6×10^8 live bacilli.

[Administration and dosage]

(1) Reconstitute the vaccine with sodium chloride injection as stated on the label. Add 1.0 ml of sodium chloride injection to the final container containing twenty human doses, and 0.5 ml to



that containing ten human doses. The vaccine shall be used up within 3 hours after reconstitution.

(2) The vaccine shall be inoculated by percutaneous scarification at the insertion of deltoid muscle area of the lateral upper arm. A dose of vaccine (0.05 ml) shall be dropped to the inoculation site and then make a scarifying mark of “#” about 1.0-1.5 cm in length with a sterile needle. The scarified place shall have a trace of oozed blood and then be pressed over with the needle for at least 10 times to let the vaccine penetrate into the skin fully. After inoculation, the arm shall remain uncovered for at least 5 minutes.

(3) For the children under the age of 14 years, the vaccine shall be applied by scarifying two marks of “#” on the skin; and, for the recipients over 14 years of age, three marks of “#” shall be scarified. It shall be 2-3 cm apart between marks.

(4) The vaccinators shall be immunized once a year.

[Adverse reactions]

The adverse reactions are mild. Infiltration at the scarified site occurs occasionally, which may not affect working in general. Transient fever occurs occasionally and can be relieved spontaneously.

[Contraindications]

The vaccine shall not be administered to the subjects with serious diseases or immunodeficiency or receiving immunosuppressive therapy, and the women in pregnancy or at the first 6 months of lactation.

[Precautions]

(1) This vaccine is only for percutaneous use and the injection is rigidly forbidden!

(2) The vaccine shall be kept away from disinfectant when the vaccine container is opened or immunization is performed.

(3) The vaccine shall not be used if any leakage of the container or illegible label is found.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

12 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;

Address;

Zip code;

Tel;

Fax;

Web site;

Anthrax Vaccine (Live) for Percutaneous Scarification

Live anthrax vaccine for percutaneous scarification is a live bacterial suspension made by the cultivation of an attenuated strain of *Bacillus anthracis* and dilution after harvest. It is used to prevent anthrax.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

The facilities for anthrax vaccine production shall be separated from other biologics production area. The apparatus for anthrax vaccine production shall be used exclusively.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The live attenuated CMCC63001 (A16R) strain used for vaccine production shall be edematous type without capsule and shall remain some residual virulence.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Control tests on seed lots

Overall tests for cultural characteristics, residual virulence, specific virulence and immunity shall be carried out on the bacterial strain every 3-5 years. Before production, the tests for morphology, cultural characteristics and phage specificity of the bacilli shall be carried out.

2.1.3.1 Cultural characteristics and biochemical reactions

The colonies of the bacilli grown on beef digest agar medium or other appropriate solid media shall be of curled hair-like appearance, opaque and greyish-white in colour. They shall be Gram-positive bacilli in chain-like array, and be able to form spores. They shall form floccules in liquid media but fail to form capsules and shall be nonmotile in serum media. They shall ferment glucose, maltose and sucrose but fail to dissociate salicin (Appendix XIV).

2.1.3.2 Tests for residual virulence

Inject s.c. 1.0 ml of the bacterial suspension of 5.0×10^7 bacilli/ml into each of five guinea pigs

weighing 350-400 g, and inject s.c. 0.1 ml of bacterial suspension of the same concentration into each of five mice weighing 18-20 g. After 10-day observation, specific deaths may occur, but only *Bacilli anthracis* without capsules shall be found by the microscopic examination of stained smears of the organs. Edema shall appear in some animals. If no edema appears in animals, the test shall be repeated. If no edema appears in the repeat test, the seed lot shall not be used for production.

2.1.3.3 Tests for specific virulence

Inject s.c. 1 ml of the bacterial suspension of 2.5×10^8 bacilli/ml into each of ten rabbits weighing 2.0-2.5 kg. All of the animals shall survive the 10-day observation period, and edema may appear at the injection sites. If any animal dies, the test shall be repeated with the same number of animals; if the deaths of animals still occur in the repeat test, the seed lot shall not be used for production.

2.1.3.4 Immunity test

Inject s.c. 1 ml of the bacterial suspension of 2.5×10^8 bacilli/ml into each of ten rabbits weighing 2.0-2.5 kg. Each rabbit shall be challenged s.c. with 20 MLD of *Bacilli anthracis* 18-20 days after injection. At the same time, inject s.c. 1 MLD of the same bacilli into each of three rabbits with the same body weight as control. After 10-day observation, all the animals in the control group shall die and not less than 60% of those in the test group shall survive.

2.1.3.5 Phage bacteriolytic test

Inoculate and spread the bacterial suspension onto the agar plates, then add one drop of *B. anthracis* phage at working concentration. After incubation at 33-34°C no growth of *B. anthracis* shall be found on the site where the phage was dropped.

2.1.4 Storage of seed lots

The seed lots shall be stored lyophilized or stored at 2-8°C in 50% glycerol solution.

2.2 Bulk

2.2.1 Working seed lots for production

Inoculate the bacterial seed onto beef digest agar medium or other appropriate media, and cultivate at 33-34°C for 18-20 hours. If no contaminating microorganisms are found, it shall be stored at 2-8°C and used within 2 weeks.

2.2.2 Production medium

The beef digest agar medium (pH 7.2-7.4) or other approved media shall be used for production.

2.2.3 Inoculation and cultivation

Inoculate the bacterial seed into a beef digest medium, and incubate at 33-34°C for 18-24 hours. The cultural characteristics, morphology and the results from the test for absence of contaminating microorganisms shall comply with the requirements given in Section 2.1.3.1.

Inoculate the seed cultures qualified in control tests onto beef digest agar medium, and cultivate at 33-34°C. More than 80% of cultures shall be typical matured spores. The cultures are harvested if it is free from contaminating microorganisms.

2.2.4 Harvest

Harvest the cultures by scraping off the bacterial lawn into the bottles containing 50% glycerol solution and make a homogenous suspension by shaking. Samples shall be taken from each bottle and tested for the absence of contaminating microorganisms (Appendix VIII A). No contaminating microorganisms shall be found in the cultures by microscopic examination of stained smears. The bacteria shall meet the characteristics of *Bacillus anthracis* CMCC63001 strain (A16R).

2.2.5 Pooling

Pool the bulks qualified in the tests for absence of contaminating microorganisms and define batches.

2.2.6 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

Dilute the bulk with sterile 50% glycerol solution to a concentration of 4.0×10^9 bacilli/ml.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

0.5 ml containing 2.0×10^9 bacilli per container for ten human doses, or 1 ml containing 4.0×10^9 bacilli per container for twenty human doses.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Tests for the absence of contaminating microorganisms

It complies with the bacterial purity test (Appendix VIII A). No contaminating microorganisms shall be found in the cultures by microscopic examination of stained smears. The bacteria shall meet the characteristics of *Bacillus anthracis* CMCC63001 (A16R) strain.

3.1.2 Bacterial content

The bacterial content shall be determined against the National Standard of Bacterial Opacity. It shall be 3.2×10^9 - 4.8×10^9 bacilli/ml.



3.2 Control tests on final bulk

Test for the absence of contaminating micro-organisms

It complies with the bacterial purity test (Appendix III A). No contaminating micro-organisms shall be found in the cultures by microscopic examination of stained smears. The bacteria shall meet the characteristics of *Bacillus anthracis* CMCC63001 (A16R) strain.

3.3 Control tests on final product

3.3.1 Identity test

See Section 2.1.3.5.

3.3.2 Inspection on final containers

The product shall be a greyish-white homogenous suspension free of foreign matters or clumps not dispersed on shaking.

3.3.3 Test for the absence of contaminating micro-organisms

It complies with the bacterial purity test (Appendix III A). No contaminating micro-organisms shall be found in the cultures by microscopic examination of stained smears. The bacteria shall meet the characteristics of *Bacillus anthracis* CMCC63001 (A16R) strain.

3.3.4 Bacterial content

The bacterial content shall be determined against the National Standard of Bacterial Opacity. It shall be 3.2×10^9 – 4.8×10^9 bacilli/ml.

3.3.5 Test for number of culturable particles

Three containers of final product are pooled and diluted to a concentration of 1.0×10^3 bacilli/ml. Inoculate 0.1 ml of the suspension onto each of five plates, spread evenly and cultivate at 35–37°C for 24 hours. Each human dose of the vaccine shall contain more than 1.0×10^8 live bacilli.

3.3.6 Potency test

The first batch of every five batches shall be sampled for the potency test according to the requirements given in Section 2.1.3.4.

3.3.7 Specific toxicity test

Inject s.c. 1 ml bacterial suspension containing 2.5×10^8 bacilli into each of five rabbits weighing 2.0–2.5 kg. All the animals shall survive the 10-day observation period, and edema at the injection site may appear. If any deaths occur, the test shall be repeated with a double number of animals. If any animal dies in the repeat test, the product shall be judged as unqualified.

4 Storage, shipping and validity period

Store and ship at 2–8°C, protected from light. The validity period is 24 months starting from the date when the test for number of culturable particles of final product proved qualified.

5 Package inserts

Directions for Use of Anthrax Vaccine (Live) for Percutaneous Scarification

[Drug name]

Adopted name: Anthrax Vaccine (Live) for Percutaneous Scarification

[Constituents and characters]

The product is a greyish-white homogenous bacterial suspension made by cultivation of a live attenuated strain of *B. anthracis* and dilution after harvest.

[Eligibles]

People living in prevalent area, those engaged in fur or leather processing, herds and those closely contacting livestock.

[Function and use]

The vaccine can induce immune response in recipients following immunization. It is used to prevent anthrax.

[Specifications]

0.5 ml containing 2.0×10^9 bacilli per container for ten human doses, or 1 ml containing 4.0×10^9 bacilli per container for twenty human doses.

[Administration and dosage]

(1) Draw the vaccine with a sterile syringe, apply two drops of the vaccine separately at two different sites, which shall be 3–4 cm apart, on the skin over the insertion of deltoid muscle of the lateral upper arm. Scarify a mark of “#” on the skin, where the vaccine was dropped, about 1.0–1.5 cm in length with a disinfected needle. The scarified places shall have a trace of oozed blood.

(2) Spread and press over the marks with the same needle repeatedly to let the vaccine penetrate into the skin fully. After inoculation the arm shall remain uncovered for at least 5–10 minutes.

(3) Repeat the inoculation if the person have no reactions at the site of scarification 24 hours after the primary inoculation.

[Adverse reactions]

Slight congestion at the injection site may occur, but no special treatment is needed. Transient low fever may occur occasionally, which can be relieved spontaneously. If the recipient develops a persistent fever or local abscess, symptomatic treatment shall be given.

[Contraindications]

The vaccine shall not be administered to the subjects with serious diseases, serious dermatosis, immunodeficiency or receiving immunosuppressive therapy or those with a history of serious allergic reaction.

[Precautions]

(1) The vaccine is only for percutaneous use, and injection is rigidly forbidden!

(2) The vaccine shall be kept away from disinfectant when the vaccine container is opened or immunization is performed.

(3) Do not use the vaccine if any leakage of container or clumps not dispersed on shaking are found.

(4) Shake the container before use. Only ethanol

but not iodine tincture can be used for disinfecting skin.

(5) The vaccine shall be used up within 3 hours after the container is opened.

(6) The containers with remaining vaccine, the used containers or apparatus shall be discarded after sterilization by boiling for 30 minutes in 3% sodium carbonate solution.

(7) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Brucellosis Vaccine (Live) for Percutaneous Scarification

Live Brucellosis vaccine for percutaneous scarification is a preparation made by the cultivation and harvest of a live attenuated strain of *Brucella abortus* and lyophilization following addition of a stabilizer. It is used to prevent brucellosis.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The live attenuated *Brucella abortus* 104 M strain shall be used for vaccine production.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

The use of bacterial strain passaged in animal is forbidden for vaccine production.

2.1.3 Control tests on seed lots

2.1.3.1 Cultural characteristics

The bacterial seeds shall grow on the medium containing 1:50000 basic fuchsin, but shall not grow on the medium containing the same concentration of thionine (alternatively tested by paper strip). A trace amount of hydrogen sulfide can be produced on liver infusion agar slant. The bacilli shall be Gram-negative coccobacilli.

2.1.3.2 Tests for dissociation

Dilute the fresh cultures into a bacterial suspension of 2.5×10^9 - 3.0×10^9 bacilli/ml with physiological saline. Put the bacterial suspension in 90°C water bath for 30 minutes, and mix the suspension at the same concentration with an equal volume of 1:1000 trypanflavine solution and place at 37°C for 24 hours. No agglutination shall be found in both cases. Examine the bacillary colonies with crystal violet staining method and the dissociation rate shall be not more than 3%.

2.1.3.3 Phage bacteriolytic test

Inoculate and spread the bacterial seed onto the liver infusion agar plate and then add one drop of brucella Tb phage. After incubation at 35-37°C for 44-48 hours, no growth of bacilli shall be found on the site where the phage was dropped.

2.1.3.4 Serological test

Dilute the fresh culture into a bacterial suspension of 5.0×10^9 bacilli/ml with physiological saline. An agglutination test shall be performed with reference brucella serum, and the agglutination titer shall be not less than the original titer of serum.

2.1.3.5 Test for residual virulence

Dilute the fresh cultures, which have been incubated on liver infusion agar slant at 35-37°C for 44-48 hours, with physiological saline into bacterial suspensions at concentrations of 1.5×10^9 , 3.0×10^9 , 6.0×10^9 , 1.2×10^{10} , and 2.4×10^{10} bacilli/ml, respectively. Divide twenty-five mice each weighing 18-20 g into five groups equally, and inject i.p. each with 0.5 ml of the bacterial suspension of the five dilutions, respectively. Observe the animals for 7 days and calculate the LD₅₀, and the LD₅₀ shall be 1.0×10^9 - 2.0×10^9 bacilli.

2.1.3.6 Immunity test

Immunity test of the bacterial seed shall be carried out at least once every 3-5 years. Dilute the fresh culture of the first passage of seed into a bacterial suspension of 2.0×10^8 bacilli/ml with physiological saline. Immunize s.c. 1 ml of the suspension into each of ten guinea pigs weighing 300-350 g. Challenge s.c. the animals with 10 or 20 MID (minimum infectious dose) of *Brucella melitensis* 25-30 days after immunization. At the same time, inject 1 MID into each of three guinea pigs in the control group. Autopsy the guinea pigs in both immunized and control groups after 25-30-day observation. Take out the lymph nodes adjacent to abdominal aorta and inguinal lymph

nodes, livers and spleens, inoculate separately onto liver infusion agar slants and incubate at 35-37°C for 10 days. If the growth of any *Brucella* is found in the cultures of the immunized animal tissues, the species and biotype shall be differentiated by using thionine medium cultivation method (or by paper strip) and hydrogen sulfide reaction method. All the three guinea pigs in the control group shall be systemically infected, that is, the *Brucella melitensis* shall be isolated from the livers or spleens. If the immunized group is challenged with 10 MID, *Brucella melitensis* shall not be isolated from more than two of the ten immunized guinea pigs. If the challenge dose is 20 MID, *Brucella melitensis* shall not be isolated from more than three of the ten immunized guinea pigs.

2.1.4 Storage of seed lots

The seed lots shall be stored lyophilized at 2-8°C.

2.2 Bulk

2.2.1 Working seed lots for production

2.2.1.1 Inoculate the bacterial seed derived from working seed lots onto liver infusion agar slant or other appropriate media and incubate at 35-37°C for 44-48 hours to prepare the first passage of seed. The first passage shall be subject to the slide agglutination test with 1 : 500 trypanflavine, and no agglutination shall be found. Only the seed with smooth surface can be used for the vaccine production. The slant inoculated with the first passage of seeds can be stored at 2-8°C for 15 days.

2.2.1.2 Inoculate the seed of the first passage onto liver infusion agar medium or other appropriate media and incubate at 35-37°C for 44-48 hours to prepare the second passage of seed. If no contaminating microorganisms are found by visual inspection, remove the condensed water and dilute the culture with sterile physiological saline into a bacterial suspension. This seed suspension shall be used as the working seed for vaccine production.

2.2.2 Production medium

The liver infusion agar medium at pH 6.6-7.2 or other approved media shall be used for production.

2.2.3 Inoculation and cultivation

The second passage of seed shall be inoculated onto the media described in Section 2.2.2 and incubated at 35-37°C for 44-48 hours. The culture bottles shall be inspected one by one and the contaminated ones shall be discarded.

2.2.4 Harvest

Harvest the cultures by washing down the bacterial lawn with the stabilizer consisting of sucrose, gelatin, thiourea and sodium glutamate, or by scraping off the bacterial lawn into the stabilizer. Cultures from several bottles can be pooled into one vessel containing the stabilizer. When control tests proved qualified, the pools can

be made into a bulk. The bulk shall be stored at 2-8°C.

2.2.5 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

The pooled bulk shall be diluted to a concentration of 1.8×10^{11} - 2.0×10^{11} bacilli/ml to prepare the final bulk. Each single human dose shall contain 9.0×10^9 - 10.0×10^9 bacilli. The final bulk qualified in the test for the absence of contaminating microorganisms can be dispensed and lyophilized. The time interval between bulk collection and lyophilization shall be not more than 7 days.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The products shall be lyophilized immediately after filling. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.4.3 Specifications

Ten human doses per container. Each single human dose contains 9.0×10^9 - 10.0×10^9 bacilli.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

Test for the absence of contaminating microorganisms

It complies with the bacterial purity test (Appendix III A). No contaminating microorganisms shall be found by microscopic examination of stained smears.

3.2 Control tests on final bulk

Test for the absence of contaminating microorganisms

It complies with the bacterial purity test (Appendix III A). No contaminating microorganisms shall be found by microscopic examination of stained smears.

3.3 Control tests on final product

Other than the determination of moisture content, the product shall be reconstituted with sodium chloride injection as stated on the label and subject to the following tests.

3.3.1 Identity test

An agglutination test shall be performed with the specific serum, and distinct agglutination shall be

observed. Alternatively, see Section 2. 1. 3. 3.

3. 3. 2 Inspection on final containers

The freeze-dried product looks like a milky-white crisp cake. The product shall be reconstituted into a homogenous suspension within 1 minute after addition of physiological saline as stated on the label.

3. 3. 3 Moisture content

The residual moisture content shall be not more than 3. 0% (Appendix VII D).

3. 3. 4 Test for the absence of contaminating microorganisms

It complies with the bacterial purity test (Appendix XII A). No contaminating microorganisms shall be found in the cultures by microscopic examination of stained smears.

3. 3. 5 Tests for bacterial species

Each sub-lot of vaccine shall be tested for the bacterial species by using thionine medium cultivation method (or by paper strip) and hydrogen sulfide reaction method. The results shall be identical to that of *Brucella abortus*.

3. 3. 6 Bacterial content

The bacterial content shall be determined against the National Standard of Bacterial Opacity. Each single human dose shall contain 9.0×10^9 - 10.0×10^9 bacilli.

3. 3. 7 Test for number of culturable particles and colony dissociation

Three containers of final products shall be sampled from every sub-lot to prepare a homogeneous bacterial suspension with physiological saline. After the opacity test, the suspension shall be diluted to a concentration of 1.0×10^3 bacilli/ml. Inoculate 0.1 ml of the suspension onto each of five plates, spread evenly with a L-shaped glass rod, and incubate at 35-37°C for 4-5 days. The number of culturable particles in the suspension shall be more than 5.0×10^9 CFU/ml. At the same time colonies shall be examined by the crystal violet colony staining method and the dissociation rate shall be not more than 10%.

3. 3. 8 Potency test

The first batch of every five batches shall be sampled for the potency test. Reconstitute the freeze-dried product with sterile physiological saline to prepare a suspension of 5.0×10^8 bacilli/ml. Immunize s. c. each of ten guinea pigs weighing 300-350 g with 1 ml of the suspension. Challenge s. c. each of the animals with 10 or 20 MID (minimum infectious dose) of *Brucella melitensis* 25-30 days after immunization. At the same time, each of three guinea pigs shall be injected s. c. with 1 MID of *Brucella melitensis* as a control. Autopsy the guinea pigs in both immunized and control groups after 25-30 day observation. Take out the lymph nodes adjacent to abdominal aorta and inguinal lymph nodes, livers and spleens, inoculate separately onto liver infusion agar slants

and incubate at 37°C for 10 days. If the growth of any *Brucella* are found on the cultures of the immunized animal tissues, the bacillary species and biotype shall be differentiated by using thionine medium cultivation method (or by paper pad) and hydrogen sulfide reaction method. All the three guinea pigs in the control group shall be systemically infected, that is, *Brucella melitensis* shall be isolated from the livers or spleens. If the immunized group is challenged with 10 MID, *Brucella melitensis* shall not be isolated from more than three of the ten immunized guinea pigs. If the challenge dose is 20 MID, *Brucella melitensis* shall not be found in more than four of the ten immunized guinea pigs.

3. 3. 9 Specific toxicity test

Three samples of final products shall be taken from every sub-lot. Inject s. c. each of five mice weighing 18-20 g with 0.5 ml of the bacterial suspension containing 1.0×10^9 bacilli/ml. The mice shall survive the 7-day observation period. If any deaths occur, the test shall be repeated. If any deaths still occur in the repeat test, the vaccine shall be discarded.

3. 3. 10 Diluent

The diluent shall be sodium chloride injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 12 months starting from the date when the test for number of culturable particles proved qualified.

5 Package inserts

Directions for Use of Brucellosis Vaccine (Live) for Percutaneous Scarification

[Drug name]

Adopted name; Brucellosis Vaccine (Live) for Percutaneous Scarification

[Constituents and characters]

The vaccine is a preparation made by the cultivation and harvest of a live attenuated strain of *Brucella abortus* and lyophilization following addition of a stabilizer. It looks like a milky-white crisp cake and shall be a homogeneous suspension after reconstitution.

[Eligibles]

The close contacts of the infectious source of brucellosis shall be immunized once a year, and the brucellin-positive people may not be immunized.

[Function and use]

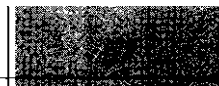
The vaccine can induce immune response in recipients following immunization. It is used to prevent brucellosis.

[Specifications]

Ten human doses per container. Each single human dose contains 9.0×10^9 - 10.0×10^9 bacilli.

[Administration and dosage]

(1) Reconstitute the vaccine of each container



with 0.5 ml of sodium chloride injection. The vaccine shall be used up within 3 hours after reconstitution, and the remaining vaccine shall be discarded.

(2) The vaccine shall be inoculated by percutaneous scarification at the insertion of deltoid muscle area of the lateral upper arm. A drop of vaccine (0.05 ml) should be applied to the inoculation area and then make a scarifying mark of “#” about 1-1.5 cm in length with a sterile needle. The scarified place should have a trace of oozed blood. The scarified place shall be pressed over with the needle for at least 10 times to let the vaccine penetrate into the skin fully. After inoculation, the arm shall remain uncovered for at least 5 minutes.

(3) For children under the age of 10 years and the persons to be revaccinated, the vaccine should be applied by scarifying one mark of “#”. For the primary vaccinees above 10 years old, two marks of “#” shall be scarified. It shall be 2-3 cm apart between marks.

[Adverse reactions]

The adverse reactions are mild. Slight infiltration at the scarified site occurs occasionally, which may not affect working in general. Transient fever occurs occasionally and can be relieved spontaneously. Any recipients who manifest symptoms suggestive to acute brucellosis caused by a wrong route of inoculation, shall be regarded as a patient with acute brucellosis and shall be treated radically.

[Contraindications]

The vaccine shall not be administered to the subjects with serious diseases or immunodeficiency, or receiving immunosuppressive therapy, and the women in pregnancy or at the first 6 months of lactation.

[Precautions]

- (1) This vaccine is only for percutaneous use, and injection is rigidly forbidden.
- (2) The vaccine shall be kept away from disinfectant when the vaccine container is opened or immunization is performed.
- (3) The vaccine shall not be used if any leakage of the container or illegible label is found.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

12 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;
Address;
Zip code;
Tel;

Fax;

Web site;

BCG Vaccine for Intradermal Injection

BCG vaccine is a freeze-dried product made by cultivation and harvest of *Bacillus of Calmette and Guerin* (BCG) and lyophilization following addition of a stabilizer. It is used to prevent tuberculosis.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

The production area for BCG vaccine shall be separated from the production area for other biologics. The apparatus for BCG vaccine production shall be used exclusively. BCG vaccine shall be protected from light at all stages of manufacture, packaging and storage.

The workers engaged in production of the product or those entering the production area frequently shall be healthy. They shall be free from tuberculosis and subject to one or two X-ray examinations of the chest each year. If suspected tuberculosis is revealed in an X-ray examination, the worker shall be kept away from BCG vaccine production temporarily.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The bacterial strain D₂PB302 shall be used for the production of BCG vaccine. The use of bacterial strains passaged in animal is forbidden for production.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lots

The total number of subcultures of a single harvest from working seed lot shall not exceed twelve passages.

2.1.4 Control tests on seed lots

2.1.4.1 Cultural characteristics

BCG shall grow well in Sauton medium at 37-39°C, which shall be acid-fast staining-positive. BCG cultivated on Sauton potato medium shall be crumpled and yellowish colonies and shall be greyish mucoid lawns on ox bile potato medium.

The BCG grown on egg-based medium shall form two types (crumpled and diffused) of yellowish colonies with protuberances. The BCG grown in Sauton medium shall form rugous and yellowish pellicles on the surface of the medium.

2.1.4.2 Virulence test

Inject i. p. 1 ml of bacterial suspension (5 mg/ml) into each of four purified protein derivative of tuberculin (TB-PPD) (i. d. injection of 0.2 ml containing 10 IU)-negative guinea pigs of the same sex, weighing 300-400 g. Weigh the animals once a week for 5 weeks, and the body weights of animals shall not decrease. The guinea pigs shall be autopsied 5 weeks after injection. Pustules on greater omentum, mesenteric lymph nodes enlargement and splenomegaly may be found. However, no lesions in liver or other organs shall be found visually.

2.1.4.3 Test for the absence of virulent mycobacteria

Inject s. c. 1 ml of bacterial suspension (10 mg/ml) at the upper medial side of thigh into each of six TB-PPD (i. d. injection of 0.2 ml containing 10 IU)-negative guinea pigs of the same sex, weighing 300-400 g. The animals shall be weighed before injection. The injection site and changes of regional lymph nodes shall be observed once a week following injection. The animals shall be weighed every 2 weeks, and the body weights of animals shall not decrease. Three guinea pigs shall be autopsied at the end of the 6th week, and the other 3 months after injection for the examination of visceral tuberculosis. No tuberculous lesions shall be found visually. If suspected tuberculous focus is found, stained smears and histological sections shall be examined microscopically. Take samples from some foci, grind and mix well with a quantity of physiological saline. The mixture shall be injected s. c. into two guinea pigs. The BCG strain shall be discarded if the tuberculous lesion is confirmed. Any animal that dies within 3 months shall be subjected to a postmortem examination. If suspected tuberculosis focus is found, the above mentioned procedures shall be performed. The BCG strain shall be discarded if the tuberculous lesion is confirmed. The test shall be repeated if non-specific death is confirmed and more than one animal die.

2.1.4.4 Immunity test

Inject s. c. 0.2 ml (one-tenth of human dose) of BCG vaccine prepared from the seed lot into each of four guinea pigs weighing 300-400g, and inject s. c. 0.2 ml of physiological saline into guinea pigs in control group. Challenge s. c. the animals with 10^3 - 10^4 virulent *Mycobacterium tuberculosis hominis* 4-5 weeks later. The animals shall be autopsied 5-6 weeks after challenge. The logarithms of pathological indexes and the number of tubercle bacillus isolated from the spleen of guinea pigs in immunized and control groups shall be analyzed

statistically, and the difference between the two groups shall be significant.

2.1.5 Storage of seed lots

The freeze-dried bacterial seeds shall be stored at 2-8°C.

2.2 Bulk

2.2.1 Production medium

Sauton potato medium, ox bile potato medium or liquid Sauton medium shall be used for production.

2.2.2 Working seed for production

The working seed subcultured once on Sauton potato medium or ox bile potato medium or in liquid Sauton medium is regarded as one passage. The storage of bacterial seed cultivated on potato media in refrigerator shall not exceed 2 months.

2.2.3 Inoculation and cultivation

Inoculate well grown pellicles onto the surface of the modified Sauton synthetic medium or other approved media, and perform static cultivation at 37°C.

2.2.4 Harvest and pooling

The culture bottles shall be examined one by one at the end of cultivation. If the contaminating micro-organism, turbidity or wet pellicles are found, the cultures shall be discarded. The pellicles shall be collected, dried by pressing, and transferred into a bottle filled with stainless steel beads. The proportion of steel beads to the bacteria shall depend upon the speed of grinder. The pellicles shall preferably be ground at a lower temperature, and diluted with a quantity of sensibiliben-free stabilizer to prepare a bulk.

2.2.5 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

The bulk shall be diluted with a stabilizer to a concentration of 1.0 mg/ml or 0.5 mg/ml.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The vaccine shall be kept in homogenous form during filling and lyophilized immediately after filling. The containers shall be sealed immediately after lyophilization.

2.4.3 Specifications

0.5 mg per container for ten human doses, or 0.25 mg per container for five human doses. 1 mg shall contain not less than 1.0×10^6 CFU of culturable particles.



2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Bacterial purity test

It complies with the bacterial purity test (Appendix VIII A). No contaminating microorganisms shall be found by microscopic examination of stained smears.

3.1.2 Bacterial content

The bacterial content shall be determined against the National Standard for Bacterial Opacity by spectrophotometry or other methods with the BCG reference preparation distributed by the NCL.

3.2 Control tests on final bulk

3.2.1 Bacterial purity test

It complies with the bacterial purity test (Appendix VIII A). No contaminating microorganisms shall be found by microscopic examination of stained smears.

3.2.2 Bacterial content

See Section 3.1.2.

3.2.3 Test for number of culturable particles

The number of culturable particles shall be not less than 1.0×10^7 CFU/mg.

3.3 Control tests on final product

Other than the tests for moisture content, the numbers of culturable particles and thermostability, the product shall be reconstituted with sterile water for injection as stated on the label and subject to the following tests.

3.3.1 Identity test

The final product shall be subject to the microscopic examination of acid-fast stained smears, and the morphology and characteristics of the bacteria shall meet those of BCG.

3.3.2 Inspection on final containers

Freeze-dried BCG looks like a white crisp cake or powder. After addition of water for injection as stated on the label, it shall turn into a homogeneous suspension within 3 minutes.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Bacterial purity test

It complies with the bacterial purity test (Appendix VIII A). No contaminating microorganisms shall be found by microscopic examination of stained smears.

3.3.5 Potency test

Each of four TB-PPD (i.d. injection of 0.2 ml containing 10 IU)-negative guinea pigs of the same sex, weighing 300-400 g, shall be injected s.c. with 0.5 mg of the sample, and shall be injected i.d. with 10 IU/0.2 ml of TB-PPD 5 weeks later. The result shall be read 24 hours after injection of TB-PPD, and the induration at the injection site

shall be not less than 5 mm in diameter.

3.3.6 Test for number of culturable particles

The culturable particles shall be counted after lyophilization on each sub-lot of batches produced from the same lot of bacterial seed, by reconstituting and pooling of five containers. The number of culturable particles of the freeze-dried vaccine after incubation for 4 weeks shall be more than 1.0×10^6 CFU/mg. Thermostability test can be performed at the same time.

3.3.7 Test for the absence of virulent mycobacteria

The test shall be performed by subcutaneous injection of a dose equivalent to 50 human doses of sample into each of six TB-PPD (i.d. injection of 0.2 ml containing 10 IU)-negative guinea pigs of the same sex, weighing 300-400 g. The animals shall be weighed every 2 weeks and observed for 6 weeks, and the body weight of animals shall not decrease. The guinea pigs shall be autopsied at the end of the 6th week for the examination of visceral tuberculosis. If no tuberculous lesion is found in liver, spleen or lung, the vaccine shall be judged as qualified. If the animals die or a suspected tuberculous focus is found, the test shall be carried out according to Section 2.1.4.3.

3.3.8 Thermostability test

Each sub-lot of vaccine shall be exposed at 37°C for 28 days and the number of culturable particles shall be compared with that of the vaccine of the same batch stored at 4°C. The number of culturable particles of the vaccine exposed at 37°C shall be not less than 2.5×10^5 CFU/mg which shall be not less than 25% of that of the vaccine stored at 4°C. Samples of vaccine shall be taken from each freeze-dryer for the test.

3.3.9 Diluent

The diluent shall be sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

Directions for Use of BCG Vaccine for Intradermal Injection

[Drug name]

Adopted name: BCG Vaccine for Intradermal Injection

[Constituents and characters]

The vaccine is a freeze-dried product made by the cultivation and harvest of BCG and lyophilization following addition of a stabilizer. It looks like a white crisp cake or powder. After reconstitution, it shall turn into a homogenous suspension.

[Eligibles]

Infants under 3 months of age and PPD (5 IU)-negative children (the induration less than 5mm in diameter 48-72 hours following PPD test).

[Function and usage]

The vaccine can induce cellular immune response in recipients following immunization. It is used to prevent tuberculosis.

[Specifications]

0.5 mg per container for ten human doses, or 0.25 mg per container for five human doses. 1 mg shall contain not less than 1.0×10^6 CFU of culturable particles.

[Dosage and administration]

(1) Add 1 ml of the accompanying diluent to the container of ten human doses, and add 0.5 ml to that of five human doses. Allow to stand for one minute and shake the container to make a homogenous suspension. The vaccine shall be used up within half an hour after reconstitution.

(2) Draw 0.1 ml of the homogenous vaccine after shaking into a sterile 1 ml syringe with blue plunger (gauge 25-26 needle), and inject i.d. at the middle area of deltoid muscle of the lateral upper arm.

[Adverse reactions]

Erythema, swelling and infiltration may occur at the injection site about 2 weeks after vaccination. If the infiltration site suppurates and ulcerates, 1% gentian violet shall be applied to prevent infection. The ulcer scab generally occurs 8-12 weeks after injection. If the regional lymph nodes enlarge, soften and form pustule, the recipient shall see a doctor in time.

[Contraindications]

The vaccine shall not be administered to the subjects with the following conditions:

- (1) Tuberculosis, acute infectious diseases, nephritis, or heart diseases.
- (2) Eczema or other dermatosis.
- (3) Immunodeficiency.

[Precautions]

- (1) Injection by subcutaneous or intramuscular route is rigidly forbidden!
- (2) Do not use the vaccine if any leakage of the container is found.
- (3) Record shall be kept in detail, including the name, sex, age and address of recipients, batch number and sub-lot number of vaccine, manufacturer and vaccination date.
- (4) In order to prevent suppurative reaction, the syringe shall be used exclusively for immunization of BCG vaccine.
- (5) The vaccine shall be protected from light even while using.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

12 months.

[Standards for Implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Diphtheria and Pertussis Combined Vaccine, Adsorbed

Adsorbed diphtheria and pertussis combined vaccine is a preparation of bulk pertussis vaccine and bulk diphtheria toxoid adsorbed onto aluminium hydroxide. It is used as a booster to prevent pertussis and diphtheria.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Monovalent bulks before mixing

2.1.1 Production of bulk pertussis vaccine shall meet the requirements given in Annex 1 of the Diphtheria, Tetanus, Pertussis Combined Vaccine, Adsorbed.

2.1.2 Production of bulk diphtheria toxoid shall meet the requirements given in Sections 2.1-2.2 of the Diphtheria Vaccine, Adsorbed.

2.1.3 Control tests on bulk

2.1.3.1 Pertussis vaccine

See Section 2 of Annex 1 of the Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed.

2.1.3.2 Diphtheria toxoid

See Section 3.1 of the Diphtheria Vaccine, Adsorbed.

2.2 Final bulk

2.2.1 Formula

The following antigens shall be contained in 1 ml of the final bulk:

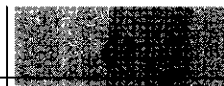
<i>Bordetella pertussis</i>	not more than 9.0×10^9 bacteria (not more than 30 IOU)
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Diphtheria toxoid	20 Lf
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2.2.2 Formulation

2.2.2.1 Dilution of aluminum hydroxide

Dilute the aluminum hydroxide with water for injection to make a final concentration of 1.0-1.5 mg/ml in the final product. Thimerosal content shall be not more than 0.1 g/L and sodium chloride content shall be made up to 8.5 g/L.



2.2.2.2 Adsorption

Add a quantity of the bulks of diphtheria toxoid and the pertussis vaccine based on the calculation into the diluted adjuvant and adjust pH to 5.8-7.2.

2.2.3 Control tests on final bulk

See Section 3.1.

2.3 Final product

2.3.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.3.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.3.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 4.0 IU of pertussis vaccine and not less than 30 IU of diphtheria toxoid.

2.3.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.2 Control tests on final product

3.2.1 Identity test

3.2.1.1 Pertussis vaccine

See Annex 2 of the Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed. The corresponding antibody shall be induced in the animals immunized with the vaccine. Alternatively, agglutination test can be carried out with the corresponding antiserum after dissolving the adjuvant with sodium citrate or sodium carbonate and separating pertussis bacteria by centrifugation. Distinct agglutination shall be observed (See Section 2.3 of Annex 1 of the Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed).

3.2.1.2 Diphtheria toxoid

Carry out the identity test by one of the following methods:

- (1) The corresponding antibody shall be induced in the animals injected with the toxoid (Appendix XI C);
- (2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);
- (3) Gel immuno-precipitation test can be carried out after adjusting pH of the toxoid to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.2 Inspection on final containers

The vaccine shall be a milky-white homogenous

suspension free of foreign matters and clumps not dispersed on shaking.

3.2.3 Chemical tests

3.2.3.1 pH

The pH shall be 5.8-7.2 (Appendix V A).

3.2.3.2 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.2.3.3 Aluminum hydroxide content

The aluminum hydroxide content shall be 1.0-1.5 mg/ml (Appendix VII F).

3.2.3.4 Thimerosal content

The thimerosal content shall be not more than 0.1 g/L (Appendix VII B).

3.2.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.2.4 Potency tests

3.2.4.1 Pertussis vaccine

See Annex 2 of the Diphtheria, Tetanus, Pertussis Combined Vaccine (Adsorbed). The potency per single human dose shall be not less than 4.0 IU and the lower 95% confidence limit shall be not less than 2.0 IU. It is allowable to repeat the test if the results fail to meet the above requirements, but the potency shall be calculated by geometric mean from the results of valid test (or by weighted geometric means when using probit analysis). The vaccine is judged as qualified if the result meets the above requirements.

3.2.4.2 Diphtheria toxoid

The potency of diphtheria toxoid per single human dose shall be not less than 30 IU (Appendix XI C).

3.2.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.6 Specific toxicity test

3.2.6.1 Pertussis vaccine

It complies with the requirements given in Annex 3 of the Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed.

3.2.6.2 Diphtheria vaccine

Inject s.c. 2.5 ml of the vaccine from each batch to each of at least four guinea pigs weighing 250-350 g at both sides of abdomen, each side 1.25 ml. Observe the animals for 30 days. Infiltrations at the injection sites may be observed, which might become indurations 5-10 days after injection and may not be completely resolved within 30 days. Weigh each animal on days 10, 20 and 30. The test is judged as qualified if the weight of each animal increases at the end of observation in comparison with that before injection, and no signs of advanced paralysis is observed.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 18 months starting from the date of adsorption. When the storage period of bulk pertussis vaccine is more than 18 months, the total validity period of the vaccine shall be not more than 36 months starting from the date of harvest.

5 Package inserts

Directions for Use of Diphtheria and Pertussis Combined Vaccine, Adsorbed

[Drug name]

Adopted name: Diphtheria and Pertussis Combined Vaccine, Adsorbed

[Constituents and characters]

This vaccine is prepared by adsorbing the bulks of pertussis vaccine and diphtheria toxoid on to aluminum hydroxide. It is a milky-white suspension containing a preservative. A precipitate may form after a long period of storage, which can be dispersed evenly on shaking.

[Eligibles]

Children aged 3 months to 6 years.

[Function and use]

The vaccine can induce immune response in recipients following immunization. It is used as a booster to prevent pertussis and diphtheria.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 4.0 IU of pertussis vaccine and not less than 30 IU of diphtheria toxoid.

[Administration and dosage]

- (1) Inject i.m. the vaccine at the buttock or in the deltoid muscle of the lateral upper arm.
- (2) The injecting dose is 0.5 ml.

[Adverse reactions]

Erythema or swelling, pain or itch may occur at the injection site. Systemic manifestation may include low fever, fatigue, headache, etc., which can be relieved spontaneously. Medical treatments should be given in time to recipients with serious reactions.

[Contraindication]

- (1) The vaccine shall not be administered to the subjects with history of epilepsy, nervous system diseases or convulsion.
- (2) Immunization shall be postponed to the subjects with acute infectious diseases (including convalescents) or with fever.
- (3) The vaccine shall not be administered to the subjects with history of allergic reactions.

[Precautions]

- (1) Shake the container before use. Do not use the vaccine if any foreign matters, leakage of container, illegible label or the clumps not dispersed on shaking are found, or the product has been frozen.
- (2) Indurations may be found at the injection site,

which may subside gradually. The second injection shall be given on the other side.

(3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(4) If abnormal conditions such as a high fever or convulsion occur after the first injection, the second injection should not be given.

(5) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

36 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed

Adsorbed diphtheria, tetanus and pertussis combined vaccine is a preparation of bulks of pertussis vaccine, diphtheria and tetanus toxoids adsorbed onto aluminum hydroxide. It is used to prevent diphtheria, tetanus and pertussis.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Monovalent bulks before mixing

2.1.1 Production of bulk pertussis vaccine shall comply with the Requirements for Bulk Pertussis Vaccine in Annex 1.

2.1.2 Production of bulk diphtheria toxoid shall comply with the requirements given in Sections 2.1-2.2 of the Diphtheria Vaccine, Adsorbed.

2.1.3 Production of bulk tetanus toxoid shall comply with the requirements given in Sections 2.1-2.2 of the Tetanus Vaccine, Adsorbed.

2.1.4 Control tests on bulk

2.1.4.1 Pertussis vaccine

See Section 2 of Annex 1.

2.1.4.2 Diphtheria toxoid

See Section 3.1 of the Diphtheria Vaccine, Adsorbed.

2.1.4.3 Tetanus toxoid

See Section 3.1 of the Tetanus Vaccine, Adsorbed.

2.2 Final bulk

2.2.1 Formula

The following antigens shall be contained in 1 ml of the final bulk:

Pertussis vaccine	$\leq 9.0 \times 10^9$ bacteria (≤ 30 IOU)
Diphtheria toxoid	20 Lf
Tetanus toxoid	5 Lf

2.2.2 Formulation

2.2.2.1 Dilution of aluminum hydroxide

Dilute the aluminum hydroxide with water for injection to make a final concentration of 1.0-1.5 mg/ml in the final product. Thimerosal content shall be not more than 0.1 g/L and sodium chloride content shall be made up to 8.5 g/L.

2.2.2.2 Adsorption

Add a quantity of the bulks of diphtheria toxoid, tetanus toxoid and the pertussis vaccine based on the calculation into the diluted adjuvant and adjust pH to 5.8-7.2.

2.2.3 Control tests on final bulk

See Section 3.1.

2.3 Final product

2.3.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.3.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.3.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 4.0 IU of pertussis vaccine, not less than 30 IU of diphtheria toxoid and not less than 40 IU (guinea pig method) or 60 IU (mouse method) of tetanus toxoid.

2.3.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.2 Control tests on final product

3.2.1 Identity test

3.2.1.1 Pertussis vaccine

Carry out the potency test according to Annex 2, and the corresponding antibody shall be induced in

the animals immunized with the vaccine. Alternatively, agglutination test can be carried out with the corresponding antiserum after dissolving the adjuvant with sodium citrate or sodium carbonate and separating pertussis bacteria by centrifugation. Distinct agglutination shall be observed. See Section 2.3 of Annex 1.

3.2.1.2 Diphtheria toxoid

Carry out the identity test by one of the following methods:

(1) The corresponding antibody shall be induced in the animals injected with the toxoid (Appendix XI C);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the toxoid to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.1.3 Tetanus toxoid

Carry out the identity test by one of the following methods:

(1) The tetanus antibody shall be induced in the animals injected with the toxoid (Appendix XI B);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the toxoid to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.2 Inspection on final containers

The vaccine shall be a homogeneous, milky-white suspension free of foreign matters and clumps not dispersed on shaking.

3.2.3 Chemical tests

3.2.3.1 pH

The pH shall be 5.8-7.2 (Appendix V A).

3.2.3.2 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.2.3.3 Aluminum hydroxide content

The aluminum hydroxide content shall be 1.0-1.5 mg/ml (Appendix VII F).

3.2.3.4 Thimerosal content

The thimerosal content shall be not more than 0.1 g/L (Appendix VII B).

3.2.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.2.4 Potency test

3.2.4.1 Pertussis component

See Annex 2. The potency of pertussis component per single human dose shall be not less than 4.0 IU

and the lower 95% confidence limit shall be not less than 2.0 IU. It is allowable to repeat the test if the results fail to meet the above requirements, but the potency shall be calculated by geometric mean from the results of valid test (or by weighted geometric mean when using probit analysis). The vaccine is judged as qualified if the results meet the above-mentioned requirements.

3.2.4.2 Diphtheria component

It complies with the potency test for diphtheria toxoid (Appendix XI C). The potency of diphtheria toxoid per single human dose shall be not less than 30 IU.

3.2.4.3 Potency test for tetanus component

It complies with the potency test for tetanus toxoid (Appendix XI B). The potency of tetanus toxoid per single human dose shall be not less than 40 IU (Appendix XI B, guinea pig method), or not less than 60 IU (Appendix XI B, mouse method).

3.2.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.6 Specific toxicity test

3.2.6.1 Pertussis component

It complies with the requirements given in Annex 3.

3.2.6.2 Diphtheria and tetanus toxoids

Inject s. c. 2.5 ml of the vaccine from each batch to each of at least four guinea pigs weighing 250-350 g at both sides of abdomen, 1.25 ml for each side. Observe the animals for 30 days. Infiltrations at the injection sites may be observed, which might become indurations 5-10 days after injection and may not be completely resolved within 30 days. Weigh each animal on days 10, 20 and 30. The test is judged as qualified if the weight of each animal increases at the end of observation in comparison with that before injection, and no local suppuration or necrosis, symptoms of tetanus or signs of advanced paralysis are observed.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 18 months starting from the date of adsorption. When the storage period of bulk pertussis vaccine is more than 18 months, the total validity period of the vaccine shall be not more than 36 months starting from the date of harvest.

5 Annexes

Annex 1 Requirements for bulk pertussis vaccine

Annex 2 Potency test for bulk pertussis vaccine

Annex 3 Toxicity test for bulk pertussis vaccine

6 Package inserts

Annex 1 Requirements for Bulk Pertussis Vaccine

This bulk is a suspension prepared by the cultivation of phase I strain of *Bordetella pertussis*, killed with a suitable bactericide, and

diluted with PBS. It is used for the production of diphtheria, tetanus and pertussis combined vaccine.

1 Manufacturing

1.1 Bacterial seed

The bacterial seed for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologicals.

1.1.1 Name and origin of bacterial strains

The bacterial strains for production shall be phase I strain of *Bordetella pertussis* containing agglutinin types 1, 2, and 3.

1.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

1.1.3 Control tests on seed lots

1.1.3.1 Cultural characteristics

The seed shall be cultivated on to Bordet-Gengou medium or other appropriate media, and shall grow with typical morphology and biochemical characteristics.

1.1.3.2 Serological test

Suspend the bacterial lawn, which has been cultivated at 35-37°C for 40-48 hours, in physiological saline or PBS to make an appropriate concentration of bacterial suspension. A quantitative agglutination test shall be performed on the suspension with reference phase I serum, and the agglutination titer shall be not less than half of the original serum titer. At the same time, carry out a qualitative agglutination test with monovalent typing serum. The type of the bacterial strain shall be identical to the stated type of the bacterium.

1.1.3.3 Skin necrosis test

Suspend the bacterial lawn, which has been cultivated for 40-48 hours, in PBS and dilute the suspension to several different concentrations. Inject i. d. the diluted suspensions into rabbits (or guinea pigs), at least two animals for each dilution, 0.1 ml per injection, and observe for 72 hours. If hemorrhagic necrosis occurs at the injection site of one of two animals injected with the suspension containing less than 4.0×10^7 bacteria, the test result shall be regarded as positive and the seed lot is qualified.

1.1.3.4 Virulence test

Carry out the test with at least three groups of mice, at least ten mice in each group, each weighing 16-18 g. Anesthetize the mice and inoculate intranasally each with 0.05 ml of the bacterial suspension derived from a bacterial lawn cultivated 20-24 hours and diluted with PBS. Observe the mice for 14 days and record the number of death. Calculate LD₅₀ according to Reed-Muench method. The number of bacteria in one LD₅₀ shall be not

more than 1.2×10^8 .

1.1.3.5 Potency test

Carry out the test according to the method given in Annex 2 after killing the bacteria with appropriate method.

1.1.4 Storage of bacterial seed

The bacterial seed shall be stored lyophilized.

1.2 Bulk

1.2.1 Working seed lot for production

Inoculate the bacterial seed derived from the working seed lots onto a modified Bordet-Gengou medium, an active carbon semisynthetic medium, or other appropriate media and incubate at 35-37°C for not more than 72 hours to prepare the first passage of working seeds, while all the passages hereafter shall be incubated for not more than 48 hours. The storage period of working seeds shall be not more than 14 days. The subculture of freeze-dried working seed lots used for production shall not exceed ten passages.

1.2.2 Cultivation

Incubate the bacterial seed with an appropriate method for not more than 48 hours. Harvest the bacteria qualified in bacterial purity test with an appropriate method and suspend in PBS pH 7.0-7.4.

1.2.3 Bacterial purity test

Take samples from each bottle (or tank) and inoculate into each tube of common agar slant and blood agar slant (or active carbon semisynthetic medium). Incubate at 35-37°C for 2 days and at 24-26°C for one day. If any contaminating microorganisms are found, the harvest shall be discarded.

1.2.4 Killing

The bacteria shall be killed with formalin at a final concentration of 0.1%. Other approved appropriate bactericides and methods for killing of bacteria may also be used.

1.2.5 Sterility test

It complies with the test for sterility (Appendix VIII A). At the same time, inoculate the samples onto common blood agar slant (or active carbon semisynthetic medium). Incubate at 35-37°C for 72 hours and at 24-26°C for 24 hours. If any growth of microorganisms is found, the harvest shall be discarded. The harvests from different seeds or prepared at different dates shall be pooled separately. A quantity of preservative can be added after pooling. Store at 2-8°C.

2 Control tests

2.1 Bacterial content

The bacterial content in each bottle of bulk vaccine shall be determined against the national standards of bacterial opacity. The dilution of bulk shall be calculated based on its concentration.

2.2 Microscopic examination of stained smears

Take samples from each bottle of bulk vaccine and

dilute to the concentration of final product for microscope examination of stained smears. The bacteria shall be Gram-negative coccobacillus. No contaminating microorganisms shall be found in at least ten fields.

2.3 Serological test

A quantitative agglutination test shall be performed on the sample from each bottle of bulk vaccine with reference phase I serum, and the agglutination titer shall be not less than half of the original serum titer. If the results fail to meet the above requirements, potency test shall be carried out. The bulk vaccine can be used if the test results are qualified. A qualitative agglutination test with monovalent typing serum shall be performed and its type shall be identical to that of the strain for bulk vaccine production.

2.4 Potency test

See Annex 2.

2.5 Sterility test

It complies with the test for sterility (Appendix VIII A).

2.6 Specific toxicity test

See Annex 3.

2.7 Storage and validity period

Bulk vaccine shall be stored at 2-8°C. The validity period of bulk vaccine is 36 months starting from the date of harvest.

Annex 2 Potency Test for Bulk Pertussis Vaccine

1 Materials

1.1 Animals

NIH mice of the same sex or equal numbers of both sexes, each weighing 10-12 g.

1.2 Reference for pertussis vaccine

The reference shall be distributed by the NCL. The reference shall be diluted to 1 IU/ml with physiological saline and further diluted 5-fold serially.

1.3 Bulk pertussis vaccine to be tested

The bulk vaccine is diluted with physiological saline to 6.0×10^8 bacteria/ml and further diluted 5-fold serially until ED_{50} falls into the dilution range.

1.4 Challenge strain and challenge bacterial suspension

1.4.1 The challenge strain is *B. pertussis* CMCC 58030 (18323).

1.4.2 Medium

Bordet-Gengou medium containing 20%-30% of sheep blood can be used.

1.4.3 Temperature and time of cultivation

The first passage of bacterial strain shall be cultivated at 35-37°C for not more than 72 hours, and can be stored at 2-8°C for 2 weeks. The challenging strain shall be cultivated for 20-24 hours.

1.4.4 Preparation of challenging bacterial suspension
Scrape the bacterial lawn which has been cultivated for 20-24 hours and proved as a pure culture by visual inspection, and suspend into physiological saline or PBS pH 7.2-7.4. Filter the suspension through sterile absorbent cotton. Determine the concentration and then dilute with casein hydrolysate pH 7.0-7.2, peptone water or broth to a concentration of 8.0×10^4 bacteria per 0.03 ml, which is used as the challenging bacterial suspension. And then make a serial dilutions of suspensions containing 8000, 800, 80 and 8 bacteria per 0.03 ml respectively. Use the above five dilutions to determine the LD_{50} of the challenging bacterial suspension.

2 Procedure

2.1 Immunization

Immunize mice of the same sex or equal numbers of both sexes with at least three dilutions of standard and sample (not more than 5-fold between two dilutions) separately, at least sixteen mice for each dilution. Inject i. p. 0.5 ml to each mouse. At the same time, fifty-five mice shall be kept and used as a control.

2.2 Challenge

At least 94% of the mice immunized with each dilution of standard or sample shall remain healthy and survive the observation period of 14-16 days after immunization. Challenge i. c. each mouse with 0.03 ml of bacterial suspension containing 8.0×10^4 bacteria by using a 0.25 ml syringe and then challenge the mice in control group to determine LD_{50} of the challenge suspension, ten mice for each dilution.

3 Result observation

At least sixteen mice are grouped on the second day of challenge. Observe the animals for 14 days and record the numbers of survival daily. The animals dying within the first 3 days are excluded from statistical analysis. On the 14th day, any animals showing signs of paralysis, head swelling, hunchback or distinct hair erection are counted as death.

4 Result calculation and evaluation

Calculate the potency of vaccine by parallel lines analysis.

The potency per single human dose shall be not less than 4.0 IU and the lower 95% confidence limit shall be not less than 2.0 IU. It is allowable to repeat the test if the results fail to meet the above requirements, but the potency shall be calculated by geometric mean from the results of valid test (or by weighted geometric mean when using probit analysis). The bulk vaccine is judged as qualified if the results meet the above-mentioned requirements.

5 Notes

5.1 Validity of the test

The test is valid provided that:

5.1.1 The ED_{50} of the standard and the sample fall into the range of the highest and the lowest immunizing doses.

5.1.2 No significant deviations are found in the parallelism and the linearity of the dose-response curves between the standard and sample.

5.1.3 One LD_{50} shall fall into the range of 100-1000 bacteria calculated by Reed-Muench method.

5.2 Test duration

The time duration shall be within 2.5 hours starting from the preparation of challenging bacterial suspension (beginning with scraping the bacterial lawn) to the injection of challenging strain into the last mouse.

Annex 3 Toxicity Test for Bulk Pertussis Vaccine

1 Materials

1.1 Animals

NIH mice each weighing 14-16 g of the same sex or equal numbers of both sexes are selected. Withhold the mice from feeding 2 hours before injection and feed as usual after injection. Weigh the total body weight of each group of animals before injection.

1.2 Sample

Bulk pertussis vaccine can be diluted with physiological saline or PBS (pH 7.2-7.4). The bacterial content shall be not less than half of a single human dose.

2 Procedure

2.1 Inject i. p. 0.5 ml of diluted bulk into each of at least ten mice of the same sex or equal numbers of both sexes for each batch.

2.2 As a control, inject i. p. 0.5 ml of physiological saline or PBS (pH 7.2-7.4) used for dilution into the mice of the same number, the same body weight and the same sex as in the test group or equal numbers of both sexes. The concentration of preservative in the control shall be the same as that for vaccine.

3 Result evaluation

3.1 Weigh the total body weight of the mice in test and control groups 72 hours and 7 days after injection respectively.

3.2 The total body weight of the mice in test group 72 hours after injection shall be not less than that before injection.

3.3 The average body weight gain of mice in test group 7 days after injection shall be not less than 60% of that in control group.

3.4 All the mice in test group shall survive.

If the result meets the above requirements, the toxicity test of the bulk is judged as qualified. If the result fails to meet the above requirements, the test may be repeated after the bulk vaccine has been stored at 2-8°C for 3-4 months. The toxicity test of the bulk is judged as unqualified if the



repeated test result fails to meet the requirements.

Directions for Use of Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed

[Drug name]

Adopted name: Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed

[Constituents and characters]

The vaccine is prepared by the adsorption of bulks of pertussis vaccine, diphtheria toxoid and tetanus toxoid on to aluminum hydroxide. It is a milky-white suspension containing preservatives. A precipitate may form after long period of storage, which can be dispersed evenly on shaking.

[Eligibles]

Children at the age of 3 months to 6 years.

[Function and use]

The product can induce immune response in recipients following immunization. It is used to prevent diphtheria, tetanus and pertussis.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 4.0 IU of pertussis vaccine, not less than 30 IU of diphtheria toxoid and not less than 40 IU (guinea pig method) or 60 IU (mouse method) of tetanus toxoid.

[Administration and dosage]

(1) The vaccine shall be injected i.m. at the buttock or in the deltoid muscle of the lateral upper arm.

(2) Primary immunization consisting of three injections shall begin at the age of 3 months and complete at the age of 12 months; 0.5 ml per injection at the intervals of 4-6 weeks. A booster of 0.5 ml shall be given at the age of 18-24 months.

[Adverse reactions]

Erythema and swelling, pain or itch may occur at the injection site. Systemic manifestation may include low fever, fatigue and headache, which can be relieved spontaneously.

Symptomatic treatment might be given in time to the subjects with severe reactions.

[Contraindications]

(1) The vaccine shall not be administered to the subjects with a history of epilepsy, nervous system diseases or convulsion.

(2) Immunization shall be postponed to the subjects with acute infectious diseases (including convalescents) or with fever.

(3) The vaccine shall not be administered to the subjects with a history of allergic reactions.

[Precautions]

(1) Shake the container before use. Do not use the vaccine if any foreign matters, leakage of container, illegible label or clumps not dispersed on shaking are found, or the product has been

frozen.

(2) Indurations may be found at the injection site, which may subside gradually. The second injection shall be given on the other side.

(3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(4) If abnormal conditions such as a high fever or convulsion occur after the first injection, the second injection should not be given.

(5) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Diphtheria, Tetanus and Acellular Pertussis Combined Vaccine, Adsorbed

Adsorbed diphtheria, tetanus and acellular pertussis combined vaccine is a preparation of bulks of acellular pertussis vaccine, tetanus toxoid and diphtheria toxoid adsorbed onto aluminum hydroxide. It is used to prevent diphtheria, tetanus and pertussis.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Monovalent bulks before mixing

2.1.1 Production of bulk acellular pertussis vaccine shall meet the requirements given in Annex 1.

2.1.2 Production of bulk diphtheria toxoid shall meet the requirements given in Sections 2.1-2.2 of the Diphtheria Vaccine, Adsorbed.

2.1.3 Production of bulk tetanus toxoid shall meet the requirements given in Sections 2.1-2.2 of the Tetanus Vaccine, Adsorbed.

2.1.4 Control tests on bulk

2.1.4.1 Control tests on bulk acellular pertussis

vaccine

See Section 2 of Annex 1.

2.1.4.2 Control tests on bulk diphtheria toxoid
See Section 3.1 of the Diphtheria Vaccine, Adsorbed.

2.1.4.3 Control tests on bulk tetanus toxoid
See Section 3.1 of the Tetanus Vaccine, Adsorbed.

2.2 Final bulk

2.2.1 Formula

The following antigens shall be contained in 1 ml of the final bulk:

Bulk acellular pertussis components	18 µg PN
Diphtheria toxoid	25 Lf
Tetanus toxoid	7 Lf

2.2.2 Preparation of adjuvant

2.2.2.1 Aluminum hydroxide can be prepared by the reaction of aluminum chloride with ammonium hydroxide or with sodium hydroxide. Remove residual ammonia by dialysis when ammonium hydroxide is used. Other appropriate methods for preparation of aluminum hydroxide can also be used.

2.2.2.2 The bulk of aluminum hydroxide shall be a light blue or milky-white colloidal suspension, free of precipitate and foreign matters.

2.2.2.3 The bulk of aluminum hydroxide shall be tested for the contents of aluminum hydroxide and sodium chloride.

2.2.3 Pooling and dilution

Add a quantity of the bulks of diphtheria toxoid, tetanus toxoid and acellular pertussis vaccine into the diluted adjuvant and adjust pH to 5.8-7.2.

2.2.4 Control tests on final bulk

See Section 3.1.

2.3 Final product

2.3.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.3.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.3.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 4.0 IU of pertussis vaccine, not less than 30 IU of diphtheria toxoid and not less than 40 IU of tetanus toxoid.

2.3.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.2 Control tests on final product

3.2.1 Identity test

3.2.1.1 Acellular pertussis antigens

The corresponding antibody shall be induced in the animals immunized with the sample (the same method as described in Section 3.2.4.1). Other appropriate antigen-antibody reaction test can also be used.

3.2.1.2 Diphtheria toxoid

Carry out the identity test by one of the following methods:

(1) The corresponding antibody shall be induced in the animals immunized with the toxoid (Appendix XI C);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the toxoid to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.1.3 Tetanus toxoid

Carry out the identity test by one of the following methods:

(1) The tetanus antibody shall be induced in the animals immunized with the toxoid (Appendix XI B);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the toxoid to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.2 Inspection on final containers

The vaccine is a milky-white homogenous suspension free of foreign matters or clumps not dispersed on shaking.

3.2.3 Chemical tests

3.2.3.1 pH

The pH shall be 5.8-7.2 (Appendix V A).

3.2.3.2 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.2.3.3 Aluminum hydroxide content

The aluminum hydroxide content shall be 1.0-1.5 mg/ml (Appendix VII F).

3.2.3.4 Thimerosal content

The thimerosal content shall be not more than 0.1 g/L (Appendix VII B).

3.2.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.2.3.6 Glutaraldehyde content

The glutaraldehyde content shall be less than 0.01 g/L (Appendix VI D).

3.2.4 Potency test

3.2.4.1 Acellular pertussis component

See Annex 2 of the Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed. The sample shall be diluted properly to the first immunizing dose and further diluted 5-fold serially. Challenge the mice 21 days after the immunization. The potency per single human dose shall be not less than 4.0 IU and the lower 95% confidence limit shall be not less than 2.0 IU. It is allowable to repeat the test if the results fail to meet the above requirements, but the potency shall be calculated by geometric mean from the results of valid test (or by weighted geometric mean when using probit analysis). The vaccine shall be judged as qualified if the result meets the above-mentioned requirements.

3.2.4.2 Diphtheria component

The potency of diphtheria toxoid per single human dose shall be not less than 30 IU (Appendix XI C).

3.2.4.3 Tetanus component

The potency of tetanus toxoid per single human dose shall be not less than 40 IU (Appendix XI B).

3.2.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.2.6 Specific toxicity test

3.2.6.1 Acellular pertussis component

See Section 2.5 of Annex.

3.2.6.2 Diphtheria and tetanus components

Inject s.c. 2.5 ml of the vaccine from each batch to each of at least four guinea pigs weighing 250-350 g at both sides of abdomen, 1.25 ml for each side. Infiltrations at the injection sites may be observed, which might become indurations 5-10 days after injection and may not be completely resolved within 30 days. Weigh each animal on days 10, 20 and 30. The test is judged as qualified if the weight of each animal increases at the end of observation in comparison with that before injection, and no local suppuration or necrosis, symptoms of tetanus or signs of advanced paralysis are observed.

3.2.7 Test for reversion to toxicity

Incubate the sample taken from each batch at 37°C for 4 weeks and then carry out the test according to the requirements given in Section 2.6 of Annex.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 24 months starting from the date of adsorption. The total validity period of the vaccine shall be not more than 36 months starting from the date of detoxification.

5 Annex

Requirements for bulk acellular pertussis vaccine

6 Package inserts

Annex Requirements for Bulk Acellular Pertussis Vaccine

The bulk consists of active ingredients including pertussis toxin (PT) and filamentous hemagglutinin (FHA) co-purified from the culture of *Bordetella pertussis* or its supernatant by ammonium sulfate fractionation and density gradient centrifugation. After detoxification, it is used for the formulation of diphtheria, tetanus and acellular pertussis combined vaccine (adsorbed).

1 Manufacturing

1.1 Bacterial seeds

The bacterial seeds for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

1.1.1 Name and origin of bacterial strains

Phase I of *Bordetella pertussis* CS strain or other appropriate strains shall be used for production.

1.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

1.1.3 Control tests on seed lot

1.1.3.1 Cultural characteristics

The seed shall be inoculated onto Bordet-Gengou medium or other appropriate media, and shall grow with typical morphology and biochemical characteristics.

1.1.3.2 Serological tests

Suspend the bacterial lawn, which has been cultivated at 35-37°C for 40-48 hours, in physiological saline or PBS to make an appropriate concentration of bacterial suspension. A quantitative agglutination test shall be performed on the suspension with reference phase I serum, and the agglutination titer shall be not less than half of the original serum titer. At the same time, carry out a qualitative agglutination test with monovalent typing serum. The type of the bacterial strain shall be identical to the stated type of the bacterium.

1.1.3.3 Skin necrosis test

Suspend the bacterial lawn, which has been cultivated for 40-48 hours, in PBS and dilute the suspension to several different concentrations. Inject i.d. the diluted suspensions into rabbits or guinea pigs, at least two animals for each dilution, 0.1 ml per injection, and observe for 72 hours. If hemorrhagic necrosis occurs at the injection site of one of two animals injected with the suspension containing less than 4.0×10^7 bacteria, the test result shall be regarded as positive and the seed lot is qualified.

1.1.3.4 Virulence test

Carry out the test with at least three groups of mice, at least ten mice in each group, each weighing 16-18 g. Anesthetize the mice and inoculate intranasally each with 0.05 ml of the bacterial suspension derived from a bacterial lawn cultivated 20-24 hours and diluted with PBS. Observe the mice for 14 days and record the number of death. Calculate LD₅₀ according to Reed-Muench method. The number of bacteria in one LD₅₀ shall be not more than 1.2×10^8 .

1.1.3.5 Potency test

See Annex 2 of the Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed. Diluted the sample to the concentration of final product, then dilute properly to the first immunizing dose and further dilute 5-fold serially. Challenge the mice 21 days after the immunization.

1.2 Bulk

1.2.1 Working seed lots for production

Inoculate the bacterial seed derived from the working seed lots onto a modified Bordet-Gengou medium or other appropriate media and incubate at 35-37°C for not more than 72 hours to prepare the first passage of working seeds, while the second passage shall be incubated for not more than 48 hours. Inoculate the second passage onto S-S (Stainer-Scholte) medium or other appropriate media and incubate at 35-37°C for not more than 48 hours, from which the working seeds for production shall be prepared. The storage period of working seeds shall be not more than 14 days. The subculture of freeze-dried working seed lots used for production shall not exceed ten passages.

1.2.2 Production medium

The approved medium shall be used for production.

1.2.3 Cultivation

Cultivation may be carried out by stationary method or by using a fermentor. Samples shall be taken for bacterial purity test during cultivation.

1.2.4 Harvest and killing

Harvest the culture at the late logarithmic growth phase or early stationary phase. Kill the bacteria by the addition of thimerosal to the cultures.

1.2.5 Purification

Remove the endotoxins by ammonium sulfate precipitation or sucrose density gradient centrifugation and collect active fractions including PT and FHA.

1.2.6 Protein nitrogen content

Carry out the determination of protein nitrogen content (Appendix VI B, method 2).

1.2.7 Purity test

Two protein bands of PT and FHA shall be mainly shown on PAGE or SDS-PAGE profile. The ratio of these two components shall be consistent from batch to batch. The active fractions including PT

and FHA shall be not less than 85% of total protein.

1.2.8 Detoxification and homogenization

Detoxify the bulk with formalin or glutaraldehyde solution and remove the detoxifying agents by appropriate methods. The bulk is obtained after ultrasonic homogenization.

2 Control tests

2.1 Microscopic examination of stained smears

Centrifuge the test sample, and examine microscopically the Gram-stained smears of the precipitate. No *Bordetella pertussis* or other bacilli shall be found.

2.2 Potency test

See Annex 2 of the Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed. Diluted the sample to the concentration of final product, then dilute properly to the first immunizing dose and further dilute 5-fold serially. Challenge the mice 21 days after the immunization.

2.3 Sterility test

It complies with the test for sterility (Appendix XII A).

2.4 Test for thermolabile toxins

Dilute the sample with physiological saline to the double concentration of final bulk. Inject i.d. 0.025 ml of the diluted sample into each of at least four suckling mice aged 48-72 hours, or 0.1 ml into each of at least two rabbits weighing 2.5 kg. Observe the animals for 4 days. No local reactions caused by thermolabile toxins shall be found.

2.5 Specific toxicity test

The toxicity reference preparation shall be diluted 3-fold serially with physiological saline, and the sample shall be diluted to the same concentration of final product. Inject i.p. each of NIH mice weighing 14-16 g with 0.5 ml of each dilution of toxicity reference and the diluted sample, at least ten mice in each group. Carry out the tests according to the methods given in Sections 2.5.1-2.5.3 separately.

2.5.1 Mouse body weight decreasing (BWD) test

Record the body weight of each mouse before and 16 hours after the injection. The BWD toxicity of the test sample shall be not more than 10 BWDU/ml upon statistical comparison of the result with that of reference.

2.5.2 Mouse leucocytosis promotion (LP) test

Collect the peripheral blood samples of the mice 3 days after injection and count leucocytes. The LP toxicity of the test sample shall be not more than 0.5 LPU/ml upon statistical comparison of the result with that of reference.

2.5.3 Mouse histamine sensitizing (HS) test

Four days after injection, each mouse shall be injected i.p. with 0.5 ml of solution containing

4 mg of histamine dihydrochloride or 2 mg of histamine diphosphate. Record the rectal temperature of each mouse 30 minutes later. The HS toxicity of the test sample shall be not more than 0.8 HSU/ml upon statistical comparison of the result with that of reference.

2.6 Test for reversion to toxicity

Take samples from each batch and incubate at 37°C for 4 weeks. Then carry out the test according to the requirements given in Section 2.5.3.

2.7 Pyrogen test

It complies with the test for pyrogen (Appendix X D). Dilute the sample with physiological saline to the 1/50 of concentration of final bulk. The injecting dose shall be 1 ml/kg of rabbit body weight.

3 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of detoxification.

Directions for Use of Diphtheria, Tetanus and Acellular Pertussis Combined Vaccine, Adsorbed

[Drug name]

Adopted name: Diphtheria, Tetanus and Acellular Pertussis Combined Vaccine, Adsorbed

[Constituents and characters]

The vaccine is prepared by the adsorption of bulks of acellular pertussis vaccine, diphtheria toxoid and tetanus toxoid onto aluminum hydroxide. It is a milky-white suspension containing preservative. A precipitate may form after a long period of storage, which can be dispersed on shaking.

[Eligibles]

Children at 3 months to 6 years of age.

[Function and use]

The product can induce immune response in recipients following immunization. It is used to prevent diphtheria, tetanus and pertussis.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 4.0 IU of pertussis vaccine, not less than 30 IU of diphtheria toxoid and not less than 40 IU of tetanus toxoid.

[Administration and dosage]

- (1) Inject i.m. the vaccine at the buttock or in the deltoid muscle of the lateral upper arm.
- (2) Primary immunization consisting of three injections shall begin at the age of 3 months and complete at the age of 12 months; 0.5 ml per injection at intervals of 4-6 weeks. A booster of 0.5 ml shall be given at the age of 18-24 months.

[Adverse reactions]

Generally, there is no adverse reaction. Occasionally, slight redness or itch at the injection site or low fever may occur, which can be relieved spontaneously. Symptomatic treatment might be

given in time to the subjects with severe reactions.

[Contraindications]

- (1) The vaccine shall not be administered to the subjects with history of epilepsy, nervous system diseases or convulsion.
- (2) Immunization shall be postponed to the subjects with acute infectious diseases (including convalescents) or with fever.
- (3) The vaccine shall not be administered to the subjects with history of allergic reactions.

[Precautions]

- (1) Shake the container before use. Do not use the vaccine if any foreign matters, leakage of container, illegible label or clumps not dispersed on shaking are found, or the product has been frozen.
- (2) Indurations may be found at the injection site, which may subside gradually. The second injection shall be given on the other side.
- (3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.
- (4) If abnormal conditions such as a high fever or convulsion occur after the first injection, the second injection should not be given.
- (5) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:
Address:
Zip code:
Tel:
Fax:
Web site:

Tetanus Vaccine, Adsorbed

Adsorbed tetanus vaccine is a preparation of purified tetanus toxoid adsorbed onto aluminum hydroxide. The toxoid is made from the toxin by the cultivation of a strain of *Clostridium tetani* in a suitable medium, and detoxified by formaldehyde and purified. The vaccine is used to prevent tetanus.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the

requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seed for production shall comply with Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

A highly toxinogenic and immunogenic strain of *Clostridium tetani* shall be used for production.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lot

The subculture of the seed from master seed lot shall not exceed five passages. The subculture of the seed from working seed lot shall not exceed ten passages.

2.1.4 Control tests on seed lot

2.1.4.1 Cultural characteristics

The bacterium is an obligate anaerobe that grows well at 37°C. When it is inoculated into cooked meat broth, the culture shall be turbid with production of gas and foul odour. The colonies grown on blood agar plate shall grow diffusely. When stab cultivation in semisolid medium has been performed, the growth shall reveal flagella motility.

2.1.4.2 Microscopic examination of stained smears

In early cultures the bacilli shall be Gram-positive rods, and spores shall be rarely seen. In 48 hour-cultures many of the bacilli shall be Gram-negative drumstick-shaped rods with spherical spores at their poles.

2.1.4.3 Biochemical reactions

The cultures fail to ferment carbohydrates. They shall liquefy gelatin, produce hydrogen sulfide, and form indole, and shall not reduce nitrate (Appendix XIV).

2.1.4.4 Test for toxin production

Inoculate bacterial seed into toxin-producing medium and sterilize the cultures by filtration. Inject 0.1 ml of the filtrate s.c. at the tail into each of at least four mice weighing 18-22 g. Observe the animals 12-24 hours after injection, and the signs including rigidity and erection of tail, hind leg tonic spasm or generalized spasm, even death shall appear.

2.1.4.5 Specific toxicity test

A quantity of filtrate from toxin-producing cultures is neutralized in vitro with diluted tetanus antitoxin. Inject 0.4 ml of the filtrate s.c. at abdomen to each of at least four mice weighing 18-22 g. At the same time the mice injected with the filtrate not neutralized with tetanus antitoxin are used as a positive control. Observe the animals

daily after injection. Apparent symptoms of tetanus and death shall be found in positive control group, while the mice in test group shall survive.

2.1.5 Storage of seed lot

The seed lot shall be preserved at 2-8°C.

2.2 Bulk toxoid

2.2.1 Toxin

2.2.1.1 Working seed for production

Bacterial seed from the working seed lot shall be subject to overall test before production, and only those qualified in the test can be used for production.

Bacterial seed from the working seed lot shall be inoculated into seed tubes containing toxin-producing medium and subcultured for two to three passages, which shall be then transplanted into seed bottles containing the same medium.

2.2.1.2 Production medium

Intensively digested casein, soybean protein or beef medium shall be used for toxin production.

2.2.1.3 Toxin production

Microbial contamination shall be avoided during the cultivation of bacteria. If any contaminating microorganisms are found in bacterial purity test by microscopic examination of stained smears, the cultures shall be discarded.

The potency of toxin in the cultures after sterilization by filtration shall be not less than 40 Lf/ml.

2.2.2 Detoxification

2.2.2.1 Detoxification of toxin or purified toxin

Add a quantity of formalin into toxin or purified toxin and incubate at an appropriate temperature for detoxification.

2.2.2.2 Detoxification test

Inoculate s.c. 500 Lf of the sample from each bottle of crude toxoid into each of at least two guinea pigs weighing 300-400 g.

Dilute the sample from each bottle of the purified toxin after detoxification with physiological saline to a concentration of 100 Lf/ml and inoculate s.c. 5 ml into each of two guinea pigs. Observe the animals on days 7, 14 and 21. No symptoms of tetanus shall be found at the end of observation period and the weight of each animal shall not decrease in comparison with that before injection. If the weight of the test animal decreases, the test shall be repeated. If the animal shows symptoms of tetanus, the toxin shall be further detoxified.

2.2.2.3 The toxoid qualified in detoxification test shall be tested for the Lf content. The toxoid shall be a clear, yellow or brownish yellow liquid.

2.2.3 Purification

2.2.3.1 Isoelectric precipitation, ultrafiltration, ammonium sulfate fractionation or other approved appropriate methods shall be used for purification



of the toxin or toxoid.

2.2.3.2 The toxoid used for purification shall be clear and free from visible contamination.

2.2.3.3 Thimerosal shall be added as a preservative at a concentration of 0.1 g/L. The purified toxoid shall be sterilized by filtration immediately.

2.2.3.4 The toxoid produced with the same bacterial strain, same medium and mixed homogeneously in one container followed by sterilization by filtration shall be defined as one batch.

2.2.4 Control tests on bulk toxoid
See Section 3.1.

2.2.5 Storage and validity period
Store at 2-8°C. The validity period is 42 months starting from the date of purification (detoxified before purification) or the date when detoxification test proved qualified (detoxified after purification).

2.3 Final bulk

2.3.1 Preparation of adjuvant

2.3.1.1 Aluminum hydroxide can be prepared by the reaction of aluminum chloride with ammonium hydroxide or with sodium hydroxide. Remove residual ammonia by dialysis when ammonium hydroxide is used.

2.3.1.2 The bulk of aluminum hydroxide shall be a light blue or milky-white colloidal suspension, free of precipitate and foreign matters.

2.3.1.3 The bulk of aluminum hydroxide shall be tested for the contents of aluminum hydroxide and sodium chloride.

2.3.2 Formulation of adsorbed toxoid

2.3.2.1 The content of tetanus toxoid shall be 7-10 Lf/ml.

2.3.2.2 The content of aluminum hydroxide shall be not more than 3.0 mg/ml.

2.3.2.3 Thimerosal can be added as a preservative at the concentration of 0.05-0.10 g/L.

2.3.3 Control tests on final bulk
See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 40 IU of tetanus toxoid.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk toxoid

3.1.1 pH

The pH shall be 6.6-7.4 (Appendix V A).

3.1.2 Determination of Lf unit

It complies with the determination of Lf unit (Appendix XI D).

3.1.3 Purity test

The purity shall be not less than 1500 Lf/mg PN.

3.1.4 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.5 Specific toxicity test

Mix equal volumes of the samples from each bottle of bulk toxoid and dilute the mixture with physiological saline to a concentration of 250 Lf/ml. Inject s.c. 2 ml of the diluted toxoid to each of four guinea pigs weighing 250-350 g and observe the animals on days 7, 14 and 21. No fester or necrosis at injection sites or symptoms of tetanus shall be found and the weight of each animal on day 21 shall increase in comparison with that before injection.

3.1.6 Test for reversion to toxicity

Dilute the samples from each bottle of bulk toxoid with PBS pH 7.0-7.4 to a concentration of 7-10 Lf/ml, respectively, and incubate at 37°C for 42 days. Inoculate s.c. 5 ml of the sample into each of four guinea pigs weighing 250-350 g and observe the animals on days 7, 14 and 21. No symptoms of tetanus shall be found and the weight of each animal on day 21 shall increase in comparison with that before injection.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

Carry out the identity test by one of the following methods:

(1) The tetanus antibody shall be induced in the animals immunized with the vaccine (Appendix XI B);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the vaccine to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.3.2 Inspection on final containers

The vaccine is a milky-white homogeneous suspension, free of foreign matters and clumps not dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Aluminum hydroxide content

The aluminum hydroxide content shall be not more than 3.0 mg/ml (Appendix VII F).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Thimerosal content

The thimerosal content shall be not more than 0.1 g/L (Appendix VII B).

3.3.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.3.4 Potency test

The potency of tetanus toxoid shall be not less than 40 IU per single human dose (Appendix XI B; guinea pig method).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Specific toxicity test

Mix equal volumes of the samples from each sublot of the vaccine. Inject s.c. 2.5 ml of the mixture into each of four guinea pigs weighing 250-350 g at abdomen. Observe the animals on days 7, 14 and 21. No fester or necrosis at the injection sites, or symptoms of tetanus shall be found and the weight of each animal on day 21 shall increase in comparison with that before injection.

3.3.7 Stability test

Stability test shall be carried out when any change of production process occurs. Final products manufactured from three consecutive batches of bulk purified toxoid shall be kept at 2-8°C for the inspection on final containers and the tests for pH, specific toxicity and potency at the end of validity period, and all the test results shall meet the requirements.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 42 months starting from the date of filling of final product.

5 Package inserts**Directions for Use of Tetanus Vaccine, Adsorbed****[Drug name]**

Adopted name: Tetanus Vaccine, Adsorbed

[Constituents and characters]

Adsorbed tetanus vaccine is a preparation of purified tetanus toxoid adsorbed onto aluminum hydroxide. The toxoid is made from the toxin by the cultivation of a strain of *Clostridium tetani* in a suitable medium and detoxified by formaldehyde and purified. The vaccine is a milky-white homogeneous suspension containing a preservative. A precipitate may form after a long period of storage,

which can be dispersed evenly on shaking, and the upper layer of the solution shall be clear and colourless.

[Eligibles]

Persons prone to be wounded are main eligibles. Pregnant women immunized with the vaccine can prevent puerperal and neonatal tetanus.

[Function and use]

The product can induce humoral immune response in recipients following immunization. It is used to prevent tetanus.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 40 IU of tetanus toxoid.

[Administration and dosage]

The vaccine shall be injected i.m. in the deltoid muscle of the lateral upper arm.

(1) Persons who have not been immunized with the tetanus toxoid shall be given three injections, each 0.5 ml, for the primary immunization. In the first year two injections should be given at an interval of 4-8 weeks and one injection give one year later. Normally one booster dose should be given every 10 years or every 5 years in particular situation.

(2) Persons who have had the primary and booster immunizations need no vaccination within 3 years from the last injection when wounded, but need a booster injection beyond 3 years from the last injection. For the persons wounded with severe contamination or without primary immunization, in addition to this vaccine, tetanus antitoxin or human tetanus immunoglobulin can be given at the other side of the injection site.

(3) Persons who have had primary immunization with combined vaccine containing tetanus toxoid shall be boosted every 10 years.

(4) For pregnant woman, the first injection can be given at the 4th month of pregnancy and the second injection at the 6th to the 7th month. The dosage for each injection is 0.5 ml.

[Adverse reactions]

Erythema and swelling, pain or itch may occur at the injection site. Systemic manifestations may include low fever, fatigue, headache, etc., which can be relieved spontaneously.

[Contraindications]

The vaccine shall not be administered to the subjects with serious diseases, fever, or with a history of allergy or of nervous system reactions following the injection of tetanus toxoid.

[Precautions]

(1) Shake the container before use. Do not use the vaccine if foreign matters, leakage of container, illegible label or clumps not dispersed on shaking are found, or the product has been frozen.

(2) Indurations may be found at the injection site, which can subside within 1 to 2 months after injection. The second injection shall be given on the other side.

(3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(4) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

42 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Diphtheria Vaccine, Adsorbed

Adsorbed diphtheria vaccine is a preparation of purified diphtheria toxoid adsorbed onto aluminum hydroxide. The toxoid is made from the toxin by the cultivation of a strain of *Corynebacterium diphtheriae* in a suitable medium and detoxified by formaldehyde and purified. The vaccine is used for children at the age of 6 months to 12 years to prevent diphtheria.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds for production shall comply with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The strain of *Corynebacterium diphtheriae* PW8 or highly toxinogenic and immunogenic strains derived from the PW8 strain shall be used for production.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lot

The subculture of the bacterial seed from master seed lot shall not exceed five passages.

2.1.4 Control tests on seed lots

2.1.4.1 Cultural characteristics

The colonies grown on Loeffler serum medium shall be circular, protuberant and grey in colour with a smooth surface and regular border; those grown on potassium tellurite agar medium shall be shining and grey-black in colour; those grown on blood agar medium shall be opaque, grey in colour, and produce no α -hemolysin.

2.1.4.2 Microscopic examination of stained smears

The bacterium shall be Gram-positive rod with metachromatic granules, and club-shaped swellings at its poles, the bacilli arrange themselves in palisades, X or Y shape.

2.1.4.3 Biochemical reactions

The cultures shall ferment glucose, maltose, galactose and dextrin with production of acid but not gas. They shall not ferment sucrose, mannitol, lactose or soluble starch (Appendix XIV).

2.1.4.4 Specific neutralization test

When the bacterial seed is inoculated onto Elek agar medium, an apparent white precipitation line shall be observed.

2.1.5 Storage of seed lot

The seed lot shall be preserved at 2-8°C.

2.2 Bulk toxoid

2.2.1 Toxin

2.2.1.1 Working seed lots for production

Bacterial seed from working seed lot shall be subject to overall test before production, and only those qualified in the test can be used for production. The bacterial seed from working seed lot shall be inoculated onto appropriate medium and subcultured in toxin-producing medium for two to three passages, which shall be then transplanted into seed bottles containing the same medium.

2.2.1.2 Production medium

Trypsin digested beef medium or other approved appropriate media shall be used for the production of diphtheria toxin.

2.2.1.3 Microbial contamination shall be avoided during toxin production. If any contaminating microorganisms are found by microscopic examination or by bacterial purity test, the cultures shall be discarded. The potency of toxin in the cultures after sterilization by filtration shall be not less than 150 Lf/ml.

2.2.2 Purification

2.2.2.1 Ammonium sulfate-active carbon fractionation method or other approved appropriate methods shall be used.

2.2.2.2 A quantity of preservative may be added in the process of dialysis. If any contaminating microorganisms are found by visual inspection, the contaminated ones shall be discarded.

2.2.2.3 Toxoid produced with the same seed, same medium, and purified by the same method, which are pooled in one container followed by sterilization by filtration shall be defined as one batch.

2.2.3 Detoxification

2.2.3.1 Add a quantity of formalin into toxin or purified toxin and incubate at an appropriate temperature for detoxification. Alternatively, lysine may be added to the purified toxin before detoxification.

2.2.3.2 Detoxification test

Dilute the samples from each bottle of toxoid or purified toxoid to a concentration of 100 Lf/ml with physiological saline separately. Inoculate i.d. 0.1 ml of the diluted samples and 25-fold dilution of Schick test toxin to each of two rabbits, weighing about 2.0 kg, separately, using 0.1 ml of sterile physiological saline as a negative control. Observe the result 96 hours after injection. The reaction at the injection site of sample shall be negative or almost undetectable, while the reaction of Schick test toxin shall be positive and that of control shall be negative.

2.2.3.3 Detoxification shall be continued if it is not complete. Additional formalin may be added if necessary.

2.2.3.4 Thimerosal can be added to toxoid as a preservative at the concentration of 0.1 g/L. Preservative may not be added in the purified toxoid prepared from purified toxin if free formaldehyde has not been removed.

2.2.4 Control tests on bulk toxoid
See Section 3.1.

2.2.5 Storage and storage period
Store at 2-8°C, protected from light. The storage period of the bulk toxoid is 42 months starting from the date when detoxification test proved qualified.

2.3 Final bulk

2.3.1 Preparation of adjuvant

2.3.1.1 Aluminum hydroxide can be prepared by the reaction of aluminum chloride with ammonium hydroxide or with sodium hydroxide. Remove residual ammonia by dialysis when ammonium hydroxide is used.

2.3.1.2 The bulk of aluminum hydroxide shall be a light blue or milky-white colloidal suspension, free of precipitate and foreign matters.

2.3.1.3 The bulk of aluminum hydroxide shall be tested for the contents of aluminum hydroxide and sodium chloride.

2.3.2 Formulation of adsorbed toxoid

2.3.2.1 The diphtheria toxoid content shall be 30-50 Lf/ml.

2.3.2.2 The aluminum hydroxide content shall be not more than 3.0 mg/ml.

2.3.2.3 Thimerosal can be added as a preservative at the concentration of 0.05-0.1 g/L.

2.3.2.4 Sodium chloride shall be added to the concentration of 7.5-9.5 g/L.

2.3.3 Control tests on final bulk
See Section 3.2

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 30 IU of diphtheria toxoid.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk toxoid

3.1.1 pH

The pH shall be 6.6-7.4 (Appendix V A).

3.1.2 Determination of flocculation unit (Lf)

It complies with the determination of flocculation unit (Appendix XI D).

3.1.3 Purity test

The purity shall be not less than 1500 Lf/mg PN.

3.1.4 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.5 Specific toxicity test

Mix equal volumes of the samples from each bottle of bulk toxoid and dilute to 50 Lf/ml with physiological saline. Inject s.c. 5 ml of the diluted toxoid at abdomen to each of four guinea pigs, weighing 250-350 g, and observe the animals for 30 days. Observe carefully the injection sites in the first 5 days, and weigh each animal on days 10, 20 and 30. Each animal shall not loss its weight continuously during the observation period. At the end of observation, the weight of each animal shall increase in comparison with that before injection. No necrosis at the injection site, desquamation, depilation or signs of advanced paralysis shall be found.

3.1.6 Test for reversion to toxicity

Dilute the samples from each bottle of purified toxoid with PBS pH 7.0-7.4 to a concentration of

30-50 Lf/ml and incubate at 37°C for 42 days. Inoculate i.d. 0.1 ml of the diluted sample and 0.1 ml of 25-fold dilution of Schick test toxin to each of two rabbits, weighing about 2 kg, separately, using 0.1 ml of PBS as negative control. Observe the result 72 hours after the injection. The diameter of erythema and swelling reactions at the injection sites of the test sample shall be less than 15 mm. The reaction of Schick test toxin shall be positive, and that of control shall be negative.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix VII A).

3.3 Control tests on final product

3.3.1 Identity test

Carry out the identity test by one of the following methods:

(1) The corresponding antibody shall be induced in the animals injected with the vaccine (the same method as that for potency test, see Section 3.3.4);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the vaccine to 9.0, and immuno-precipitation reaction shall be observed (Appendix VII C).

3.3.2 Inspection on final containers

The vaccine is a milky-white homogeneous suspension free of foreign matters and clumps not dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Aluminum hydroxide content

The aluminum hydroxide content shall be not more than 3.0 mg/ml (Appendix VII F).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Thimerosal content

The thimerosal content shall be not more than 0.1 g/L (Appendix VII B).

3.3.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI K).

3.3.4 Potency test

The potency of diphtheria toxoid shall be not less than 30 IU per single human dose (Appendix XI C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix VII A).

3.3.6 Specific toxicity test

Mix equal volumes of samples from each sub-lot of the vaccine and inoculate s.c. 2.5 ml of the mixture into each of four guinea pigs weighing 250-350 g at abdomen. Observe the animals for 30 days. Infiltrations at the injection sites may be observed which might become indurations 5 to 10 days after injection and may not be completely absorbed within 30 days. Weigh each animal on days 10, 20 and 30. The test is judged as qualified if the weight of each animal increases at the end of observation in comparison with that before injection and no signs of advanced paralysis are observed.

3.3.7 Stability test

Stability test shall be carried out when any change of production process occurs. The samples of final products from three consecutive batches of bulk purified toxoid shall be kept at 2-8°C for the inspection on final containers and the tests for pH, specific toxicity and potency at the end of validity period. All the test results shall meet the requirements.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Diphtheria Vaccine, Adsorbed

[Drug name]

Adopted name: Diphtheria Vaccine, Adsorbed

[Constituents and characters]

Adsorbed diphtheria vaccine is a preparation of purified diphtheria toxoid adsorbed onto aluminum hydroxide. The toxoid is made from the toxin by the cultivation of a strain of *Corynebacterium diphtheria* in a suitable medium and detoxification by formaldehyde and purification. The vaccine is a milky-white homogeneous suspension containing a preservative. A precipitate may form after a long period of storage, which can be dispersed on shaking.

[Eligibles]

Children at the age of 6 months to 12 years.

[Function and use]

The product can induce humoral immune response in recipients following immunization. It is used for children at the age of 6 months to 12 years to prevent diphtheria.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml, or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 30 IU of diphtheria toxoid.

[Administration and dosage]

(1) The vaccine shall be injected i.m. in the deltoid muscle of the lateral upper arm.

(2) Three injections of 0.5 ml each should be

given for the primary immunization. In the first year two injections should be given at an interval of 4-8 weeks and one injection given one year later. One booster dose of 0.5 ml should be administered 3-5 years after the complete course of primary immunization.

[Adverse reactions]

Erythema and swelling, pain or itch may occur at the injection site. Systemic manifestation may include low fever, fatigue, headache, etc., which can be relieved spontaneously.

[Contraindications]

The vaccine shall not be administered to the subjects with serious diseases, fever, or with a history of allergy or of nervous system reactions following the injection of diphtheria toxoid.

[Precautions]

(1) Shake the container before use. Do not use the vaccine if foreign matters, leakage of container, illegible label or any clumps not dispersed on shaking are found, or the product has been frozen.

(2) Indurations may be found at the injection site, which may subside within 1 to 2 months after injection. The second injection shall be given on the other side.

(3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(4) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light. Prevent freezing.

[Packaging]

[Validity period]

36 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;
Address;
Zip code;
Tel;
Fax;
Web site;

Diphtheria Vaccine for Adults and Adolescents, Adsorbed

Adsorbed diphtheria vaccine for adults and adolescents is a preparation of bulk diphtheria toxoid adsorbed onto aluminum hydroxide. The vaccine is used as a booster for adults and adolescents who have received a primary immunization of diphtheria vaccine. It is also used for

an emergent immunization.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Bacterial seeds

See Section 2.1 of the Diphtheria Vaccine, Adsorbed.

2.2 Bulk

2.2.1 Preparation of bulk

See Section 2.2 of the Diphtheria Vaccine, Adsorbed.

2.2.2 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Preparation of adjuvant

2.3.1.1 Aluminum hydroxide can be prepared by the reaction of aluminum chloride with ammonium hydroxide or with sodium hydroxide. Remove residual ammonia by dialysis when ammonium hydroxide is used.

2.3.1.2 The bulk of aluminum hydroxide shall be a light blue or milky-white colloidal suspension, free of precipitate and foreign matters.

2.3.1.3 The bulk of aluminum hydroxide shall be tested for the contents of aluminum hydroxide and sodium chloride.

2.3.2 Formulation of adsorbed toxoid

2.3.2.1 The content of bulk diphtheria toxoid shall be 4 Lf/ml, and its purity shall be not less than 2000 Lf/mg PN.

2.3.2.2 The aluminum hydroxide content shall be not more than 2.5 mg/ml.

2.3.2.3 Thimerosal can be added as a preservative at a concentration of 0.05-0.1 g/L.

2.3.3 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 2 IU of diphtheria toxoid.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk toxoid

3.1.1 pH

The pH shall be 6.6-7.4 (Appendix V A).

3.1.2 Determination of flocculation unit (Lf)

It complies with the determination of flocculation unit (Appendix XI D).

3.1.3 Purity

The purity shall be not less than 2000 Lf/mg PN.

3.1.4 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.5 Specific toxicity test

Mix equal volumes of samples from each bottle of bulk toxoid and dilute to 50 Lf/ml with physiological saline. Inject s.c. 5 ml of the diluted toxoid to each of four guinea pigs weighing 250-350 g at abdomen, and observe the animals for 30 days. Observe carefully the injection sites in the first 5 days, and weigh each animal on days 10, 20 and 30. Each animal shall not lose its weight continuously during the observation period. At the end of observation, the weight of each animal shall increase in comparison with that before injection. No necrosis at the injection site, desquamation, depilation or signs of advanced paralysis shall be found.

3.1.6 Test for reversion to toxicity

Dilute the samples from each bottle of bulk toxoid with PBS pH 7.0-7.4 to a concentration of 30-50 Lf/ml and incubate at 37°C for 42 days. Inoculate i.d. 0.1 ml of the diluted sample and 0.1 ml of 25-fold dilution of Schick test toxin to each of two rabbits, weighing about 2 kg, separately, using 0.1 ml of PBS as negative control. Observe the result 72 hours after the injection. The diameter of erythema and swelling reactions at the injection sites of the test sample shall be less than 15 mm. The reaction of Schick test toxin shall be positive, and that of control shall be negative.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

Carry out the identity test by one of the following methods;

(1) The corresponding antibody shall be induced in the animals injected with the vaccine (the same method as that for potency test, see Section 3.3.4);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the vaccine to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.3.2 Inspection on final containers

The vaccine is a milky-white homogeneous suspension free of foreign matters and clumps not dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Aluminum hydroxide content

The aluminum hydroxide content shall be not more than 2.5 mg/ml (Appendix VII F).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Thimerosal content

The thimerosal content shall be not more than 0.10 g/L (Appendix VII B).

3.3.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.3.4 Potency test

The potency of diphtheria toxoid shall be not less than 2 IU per single human dose (Appendix XI C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Specific toxicity test

Mix equal volumes of samples from each sub-lot of the vaccine and inoculate s.c. 2.5 ml of the mixture to each of four guinea pigs, weighing 250-350 g. Observe the animals for 30 days. Infiltration at the injection sites may be observed which might become indurations 5 to 10 days after injection and may not be completely absorbed within 30 days. Weigh each animal on days 10, 20 and 30. The test is judged as qualified if the weight of each animal increases at the end of observation in comparison with that before injection and no signs of advanced paralysis are observed.

3.3.7 Stability test

Stability test shall be carried out when any change of production process occurs. Final products manufactured from three consecutive batches of bulk purified toxoid shall be kept at 2-8°C for the inspection on final containers and the test for pH, specific toxicity and potency at the end of validity period. All the test results shall meet the requirements.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Diphtheria Vaccine for Adults and Adolescents, Adsorbed

[Drug name]

Adopted name: Diphtheria Vaccine for Adults and Adolescents, Adsorbed

[Constituents and characters]

Adsorbed diphtheria vaccine for adults and adolescents is a preparation of bulk diphtheria toxoid adsorbed onto aluminum hydroxide. The vaccine is a milky-white homogeneous suspension containing a preservative. A precipitate may form after a long period of storage, which can be dispersed on shaking.

[Eligibles]

People older than 12 years of age.

[Function and use]

The product can induce humoral immune response in recipients following immunization. It is used as a booster for adults and adolescents who have received primary immunization of diphtheria vaccine. It is also used for an emergent immunization.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 2 IU of diphtheria toxoid.

[Administration and dosage]

- (1) The vaccine should be injected i.m. in the deltoid muscle of the lateral upper arm.
- (2) Dosage: One single injection of 0.5 ml.

[Adverse reactions]

- (1) Erythema, swelling, indurations, pain or itch may occur at the injection sites. Systemic manifestations may include low fever, fatigue, headache, etc., which can be relieved spontaneously.
- (2) Indurations may be found at the injection site, which may subside within 1-2 months after injection.

[Contraindications]

The vaccine shall not be administered to the subjects with serious diseases, fever, or with a history of allergic or of nervous system reactions following the injection of diphtheria toxoid.

[Precautions]

- (1) Shake the container before use. Do not use the vaccine if any foreign matters, leakage of container, illegible label or clumps not dispersed on shaking are found, or the product has been frozen.
- (2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.
- (3) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

36 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Diphtheria and Tetanus Combined Vaccine, Adsorbed

Adsorbed diphtheria and tetanus combined vaccine is a preparation of bulk diphtheria and tetanus toxoids adsorbed onto aluminum hydroxide. It is used as a booster for children who have received primary immunization of pertussis, diphtheria and tetanus combined vaccine.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Monovalent bulks before mixing

2.1.1 Production of bulk diphtheria toxoid shall meet the requirements given in the Sections 2.1-2.2 of the Diphtheria Vaccine, Adsorbed.

2.1.2 Production of bulk tetanus toxoid shall meet the requirements given in the Sections 2.1-2.2 of the Tetanus Vaccine, Adsorbed.

2.1.3 Control tests on bulk

See Section 3.1 of the Diphtheria Vaccine, Adsorbed and the Tetanus Vaccine, Adsorbed.

2.2 Final bulk

2.2.1 Formulation

2.2.1.1 Formula

The following antigens shall be contained in 1 ml of the final bulk:

Bulk diphtheria toxoid	not more than 20 Lf
Bulk Tetanus toxoid	not more than 3 Lf

2.2.1.2 Preparation of adjuvant

(1) Aluminum hydroxide can be prepared by the reaction of aluminum chloride with ammonium hydroxide or with sodium hydroxide. Remove residual ammonia by dialysis when ammonium hydroxide is used.



(2) The bulk of aluminum hydroxide shall be a light blue or milky-white colloidal suspension, free of precipitate and foreign matters.

(3) The bulk of aluminum hydroxide shall be tested for the contents of aluminum hydroxide and sodium chloride. The aluminum hydroxide content shall be not more than 3.0 mg/ml.

2.2.1.3 The sodium chloride content shall be 8.5 g/L.

2.2.1.4 Thimerosal can be added as a preservative at the concentration of 0.05-0.10 g/L.

2.2.2 Control test on final bulk

See Section 3.1.

2.3 Final product

2.3.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.3.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.3.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 30 IU of diphtheria toxoid and not less than 40 IU of tetanus toxoid.

2.3.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.2 Control tests on final product

3.2.1 Identity test

3.2.1.1 Diphtheria toxoid

Carry out the identity test by one of the following methods:

(1) The corresponding antibody shall be induced in the animals injected with the vaccine (the same method as that for potency test, see Section 3.2.4.1);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the vaccine to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.1.2 Tetanus toxoid

Carry out the identity test by one of the following methods:

(1) The tetanus antibody shall be induced in the animals injected with the vaccine (Appendix XI B);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the vaccine to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.2 Inspection on final containers

The vaccine is a milky-white homogeneous suspension, free of foreign matters and clumps not dispersed on shaking.

3.2.3 Chemical tests

3.2.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.2.3.2 Aluminum hydroxide content

The aluminum hydroxide content shall be not more than 2.5 mg/ml (Appendix VII F).

3.2.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.2.3.4 Thimerosal content

The thimerosal content shall be not more than 0.1 g/L (Appendix VII B).

3.2.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.2.4 Potency test

3.2.4.1 Diphtheria toxoid

The potency of diphtheria toxoid shall be not less than 30 IU per single human dose (Appendix XI C).

3.2.4.2 Tetanus toxoid

The potency of tetanus toxoid shall be not less than 40 IU per single human dose (Appendix XI B).

3.2.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.6 Specific toxicity test

Mix equal volumes of samples from each sub-lot of the vaccine and inoculate s.c. 2.5 ml of the mixture to each of four guinea pigs weighing 250-350 g at abdomen. Observe the animals for 30 days. Infiltrations at the injection sites may be observed which may become indurations 5 to 10 days after injection and may not be completely absorbed within 30 days. Weigh each animal on days 10, 20 and 30. The test is judged as qualified if the weight of each animal increases at the end of observation period in comparison with that before injection and no fester or necrosis at the injection sites, signs of advanced paralysis or symptoms of tetanus are observed.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light.



The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Diphtheria and Tetanus Combined Vaccine, Adsorbed

[Drug name]

Adopted name: Diphtheria and Tetanus Combined Vaccine, Adsorbed

[Constituents and characters]

Adsorbed diphtheria and tetanus combined vaccine is a preparation of bulk diphtheria and tetanus toxoids adsorbed onto aluminum hydroxide. The vaccine is a milky-white homogeneous suspension containing thimerosal as a preservative. A precipitate may form after a long period of storage, which can be dispersed on shaking.

[Eligibles]

Children younger than 12 years of age.

[Function and use]

The product can induce immune response in recipients following immunization. It is used as a booster for children who have received primary immunization of pertussis, diphtheria and tetanus combined vaccine.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml, or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 30 IU of diphtheria toxoid and not less than 40 IU of tetanus toxoid.

[Administration and dosage]

(1) The vaccine shall be injected i.m. in the deltoid muscle of the lateral upper arm.

(2) Dosage: One single injection of 0.5 ml.

[Adverse reactions]

Erythema and swelling, pain or itch may occur at the injection site. Systemic manifestation may include low fever, fatigue, headache, etc., which can be relieved spontaneously. Indurations may be found at the injection site, which may subside within 1-2 months after injection.

[Contraindications]

The vaccine shall not be administered to the subjects with severe diseases, fever, or with a history of allergy or of nervous system reactions following the injection of diphtheria or tetanus toxoid.

[Precautions]

(1) Shake the vaccine before use. Do not use the vaccine if any foreign matters, leakage of container, illegible label or clump not dispersed on shaking are found, or the product has been frozen.

(2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(3) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

36 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Diphtheria and Tetanus Combined Vaccine for Adults and Adolescents, Adsorbed

Adsorbed diphtheria and tetanus combined vaccine for adults and adolescents is a preparation of bulk diphtheria and tetanus toxoids adsorbed onto aluminum hydroxide. The vaccine is used as a booster for adults and adolescents who have received primary immunization of diphtheria and tetanus vaccine and is used for an emergent immunization of diphtheria.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Monovalent bulks before mixing

2.1.1 Production of bulk diphtheria toxoid shall meet the requirements given in Sections 2.1-2.2 of the Diphtheria Vaccine, Adsorbed.

2.1.2 Production of bulk tetanus toxoid shall meet the requirements given in Sections 2.1-2.2 of the Tetanus Vaccine, Adsorbed.

2.1.3 Control tests on bulk

See Section 3.1 of the Diphtheria Vaccine, Adsorbed and the Tetanus Vaccine, Adsorbed.

2.2 Final bulk

2.2.1 Formulation of final bulk

2.2.1.1 Formula

The following antigens shall be contained in 1 ml of the final bulk:

Bulk diphtheria toxoid	not more than 4 Lf
Bulk tetanus toxoid	not more than 5 Lf

2.2.1.2 Preparation of adjuvant

(1) Aluminum hydroxide can be prepared by the reaction of aluminum chloride with ammonium

hydroxide or with sodium hydroxide. Remove residual ammonia by dialysis when ammonium hydroxide is used.

(2) The bulk of aluminum hydroxide shall be a light blue or milky-white colloidal suspension, free of precipitate and foreign matters.

(3) The bulk of aluminum hydroxide shall be tested for the contents of aluminum hydroxide and sodium chloride. The aluminum hydroxide content shall be not more than 3.0 mg/ml.

2.2.1.3 The sodium chloride content shall be 7.5-9.5 g/L.

2.2.1.4 Thimerosal can be added as a preservative at the concentration of 0.05-0.10 g/L.

2.2.2 Control tests on final bulk

See Section 3.1.

2.3 Final product

2.3.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.3.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.3.3 Specifications

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 2 IU of diphtheria toxoid and not less than 40 IU of tetanus toxoid.

2.3.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix VIII A).

3.2 Control tests on final product

3.2.1 Identity test

3.2.1.1 Diphtheria toxoid

Carry out the identity test by one of the following methods;

(1) The corresponding antibody shall be induced in the animals injected with the vaccine (the same method as that for potency test, see Section 3.2.4.1);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the vaccine to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.1.2 Tetanus toxoid

Carry out the identity test by one of the following methods;

(1) The tetanus antibody shall be induced in the animals injected with the vaccine (Appendix XI B);

(2) Flocculation test can be carried out after dissolving the adjuvant with sodium citrate or sodium carbonate, and flocculation shall be observed (Appendix XI D);

(3) Gel immuno-precipitation test can be carried out after adjusting pH of the vaccine to 9.0, and immuno-precipitation reaction shall be observed (Appendix VIII C).

3.2.2 Inspection on final containers

The vaccine is a milky-white homogeneous suspension, free of foreign matters and clumps not dispersed on shaking.

3.2.3 Chemical tests

3.2.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.2.3.2 Aluminum hydroxide content

The aluminum hydroxide content shall be not more than 2.5 mg/ml (Appendix VII F).

3.2.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.2.3.4 Thimerosal content

The thimerosal content shall be not more than 0.1 g/L (Appendix VII B).

3.2.3.5 Free formaldehyde content

The free formaldehyde content shall be not more than 0.2 g/L (Appendix VI L).

3.2.4 Potency test

3.2.4.1 Diphtheria toxoid

The potency of diphtheria toxoid shall be not less than 2 IU per single human dose (Appendix XI C).

3.2.4.2 Tetanus toxoid

The potency of tetanus toxoid shall be not less than 40 IU per single human dose (Appendix XI B).

3.2.5 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.2.6 Specific toxicity test

Mix equal volumes of samples from each sub-lot of the vaccine and inoculate s.c. 2.5 ml of the mixture to each of four guinea pigs weighing 250-350 g at abdomen. Observe the animals for 30 days. Infiltration at the injection sites may be observed which might form indurations 5 to 10 days after injection and may not be completely resolved within 30 days. Weigh each animal on days 10, 20 and 30. The test is judged as qualified if the weight of each animal increases at the end of observation period in comparison with that before injection and no purulence or necrosis at the injection site, symptoms of tetanus or signs of

advanced paralysis are observed.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

Directions for Use of Diphtheria and Tetanus Combined Vaccine for Adults and Adolescents, Adsorbed

[Drug name]

Adopted name: Diphtheria and Tetanus Combined Vaccine for Adults and Adolescents, Adsorbed

[Constituents and characters]

Adsorbed diphtheria and tetanus combined vaccine for adults and adolescents is a preparation of bulk diphtheria and tetanus toxoids adsorbed onto aluminum hydroxide.

The vaccine is a milky-white homogeneous suspension containing thimerosal as a preservative. A precipitate may form after a long period of storage, which can be dispersed on shaking, and the upper layer of the solution shall be clear and colourless.

[Eligibles]

People older than 12 years of age.

[Function and use]

The product can induce humoral immune response in recipients following immunization. It is used as a booster for people over 12 years of age who have received primary immunization of diphtheria and tetanus vaccines. It is also used for an emergent immunization against diphtheria.

[Specifications]

0.5 ml, 1.0 ml, 2.0 ml or 5.0 ml per container. Each single human dose is 0.5 ml containing not less than 2 IU of diphtheria toxoid and not less than 40 IU of tetanus toxoid (guinea pig method).

[Administration and dosage]

(1) The vaccine should be injected i.m. in the deltoid muscle of the lateral upper arm.

(2) Dosage: One single injection of 0.5 ml.

[Adverse reactions]

Erythema and swelling, pain or itch may occur at the injection site. Systemic manifestation may include low fever, fatigue, headache, etc., which can be relieved spontaneously. Indurations may be found at the injection site, which may subside within 1-2 months after injection.

[Contraindications]

The vaccine shall not be administered to the subjects with serious diseases, fever, or with a history of allergic or of nervous system reactions following the injection of diphtheria or tetanus toxoid.

[Precautions]

(1) Shake the container before use. Do not use

the vaccine if any foreign matters, leakage of container, illegible label or clumps not dispersed on shaking are found, or the product has been frozen.

(2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(3) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

36 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Japanese Encephalitis Vaccine, Inactivated

Japanese encephalitis (JE) inactivated vaccine is a preparation of JE virus grown on primary hamster kidney cell cultures. After cultivation and harvest, the virus suspension is inactivated to make the vaccine. It is used to prevent Japanese encephalitis.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Primary hamster kidney cells or those subcultured continuously for not more than five passages can be used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

Hamsters at about 2 weeks of age are selected. Extract and mince the kidneys aseptically. Digest the tissue fragments with a quantity of trypsin. Disperse the cells with culture medium to prepare cell suspension that is then distributed into bottles

and incubated at 37°C.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

P₃ strain of JE virus shall be used as the seed for the production of the vaccine.

2.2.2 Establishment of seed lot system

The Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics shall apply.

Master seed lots and working seed lots shall be prepared by inoculating i. c. mice or suckling mice. The primary seed lot shall not exceed the 53th passages. The master seed lot shall not exceed the 68th passage. The working seed lot shall not exceed the 69th passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Mix the virus from seed lot with an equal amount of the corresponding specific immune serum. Keep the virus-immune serum mixture in 37°C water bath for 90 minutes. Neutralization test shall be conducted by intracerebral inoculation in mice to verify the specificity. The neutralization index shall be more than 500.

2.2.3.2 Virus titration

Mice each weighing 7-9 g shall be used for virus titration by intracerebral route. The titers of the seed lots shall be not less than 9.0 lg LD₅₀/ml.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix III A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix III C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the master seed virus to immunize ten mice each weighing 12-14 g. Each mouse receives 0.3 ml i. p. twice at an interval of 3 days. Ten to fourteen days after the first inoculation, challenge the immunized mice i. p. with a quantity of virus (P₃ strain not for production) suspension containing not less than 10000 LD₅₀. The protective rate of immunized group shall be 100% and the mortality of control group shall be not less than 80%.

2.2.4 Storage of virus seeds

Virus seeds shall be stored at or below -60°C; the liquid ones shall be stored for a period not more than one year.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Earle solution containing lacto-albumin hydrolysate and a quantity of inactivated calf serum shall be used as the culture medium. Other suitable culture media can also be used. The quality of calf serum free from JE antibody shall comply with the related requirements (Appendix III D).

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix III C).

2.3.4 Virus inoculation and incubation

Bottles with confluent cell sheet are selected. After rinsing the cell sheets thoroughly with PBS to remove calf serum, a quantity of maintenance medium shall be added. Inoculate virus and incubate the bottles at a suitable temperature for a period of time.

2.3.5 Virus harvest

Harvest the virus suspensions from bottles, in which the cell cultures manifest typical CPE microscopically. The harvests are collected into a large container to make the harvest virus suspension homogeneously. The final clarification shall be implemented by means of membrane filtration or continuous flow centrifugation. However, if the cell quality is good enough after harvest, fresh maintenance medium can be added again for further cultivation for multiple harvests.

2.3.6 Control tests on virus harvests

See Section 3.1.

2.3.7 Virus inactivation

Formalin shall be added to the virus harvest at a proportion of 1 : 2000 to inactivate the virus at an appropriate temperature for a period of time. The inactivated viral suspension is defined as the bulk. Thimerosal can be added and its final concentration shall be not more than 0.10 mg/ml.

2.3.8 Control tests on bulk

See Section 3.2.

2.4 Final bulk

2.4.1 Formulation

A number of bulk which passed the sterility test can be pooled together to make the final bulk.

2.4.2 Control tests on final bulk

See Section 3.3.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

2.0 ml, 5.0 ml or 10 ml per container. 0.5 ml per single human dose.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on virus harvests

3.1.1 Virus titration

Take a sample from the pooled harvest containing not more than 150 liters of virus suspension for virus titration. Dilute the test sample 10-fold serially. Inoculate i. c. each mouse of 7-9 g with 0.03 ml of at least three consecutive dilutions. Four mice are employed for each dilution. Observe the mice daily for 14 days. The mice that die within 3 days after inoculation shall be excluded in evaluation. The preparation is judged as qualified with a titer of not less than $7.5 \lg LD_{50}/ml$.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control tests on bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.2 Validation test for effective inactivation

Take a sample from the bulk for validation test for effective inactivation of virus, each sample shall represent a batch of bulk containing not more than 150 liters of virus suspension. Inoculate i. c. 0.03 ml of the test sample into each of eight mice weighing 12-14 g and concurrently inoculate i. p. 0.5 ml of the sample into each of the animals. This is the first passage in animals for the assumed virus living in the sample. Seven days after the inoculation, kill three mice of the first passage. Emulsify the brains taken from the killed mice to make 10% suspension that is used to inoculate i. c. into other six mice with the same method as mentioned above. It is the second passage. Seven days later, three mice of the second passage are killed to prepare brain suspension again as mentioned above. Inoculate the brain suspension of the second passage into other six mice to make the third passage. Observe all the mice daily for 14 days from the date of injections. The test passes if all the mice of the three passages survive the observation periods except those killed from every passage and those die within 3 days after injection due to non-specific causes.

If individual mouse dies 3 days after inoculation, the brain shall be removed immediately and emulsified to make a suspension that is used to

inoculate i. c. into other three mice. Observe the mice daily for 14 days. The test passes if the inoculated mice survive the observation period. If some mice die, the test shall be repeated. If the result of repeated test passes, this batch of bulk is still judged as qualified. If there are still deaths of mice, this batch of bulk shall be discarded.

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.4 Control tests on final product

3.4.1 Identity test

See Section 3.4.4. The test shall be considered unqualified if the potency test fails.

3.4.2 Inspection on final containers

The vaccine shall be a clear, orange-red liquid free of foreign matters and precipitate.

3.4.3 Chemical tests

3.4.3.1 pH

The pH shall be 7.2-8.0 (Appendix V A).

3.4.3.2 Content of free formaldehyde

The content of free formaldehyde content shall be not more than 0.50 mg/ml (Appendix VI L).

3.4.3.3 Thimerosal content

The thimerosal content shall be not more than 0.10 mg/ml (Appendix VII B).

3.4.4 Potency test

Test sample shall be taken from the bulk for potency test which is conducted for determination of neutralizing antibody in mice. Each sample for the test shall represent a batch of virus suspension of not more than 1000 liters. Plaque-reduction assay is employed. The reference vaccines (RA and RB) and positive serum used in the test shall be provided by the NCL.

Dilute the test vaccine (T) and reference vaccines (RA and RB) to 1:32, respectively. At an interval of 7 days, inject i. p. two doses of 0.5 ml of each diluted vaccine, respectively into each of ten mice weighing 12-14 g. Seven days following the second dose animals are bled individually. The separated sera from each mouse in the same group are pooled in an equal quantity and then inactivated at 56°C for 30 minutes. The diluted positive serum, the diluted serum against the test vaccine, and the diluted sera against reference vaccine are mixed respectively with an equal volume of diluted challenge virus (containing about 200 PFU/0.4 ml). Meanwhile, the diluted virus suspension is mixed with an equal volume of serum from healthy mice as the virus control. All the mixtures and the virus control suspension shall be kept in 37°C water bath for 90 minutes. Afterwards, 0.4 ml aliquots of the neutralized mixtures as well as the virus control are dispensed separately into each well of 6-well microplates with BHK₂₁ cells. Overlaid with medium containing methylcellulose

in the wells with virus-serum mixtures as well as the virus control. The plates are then incubated in carbon dioxide incubator at 37°C for 90 minutes. The cells in the wells are stained after incubation for 5 days and the plaques formed are counted. The plaque reduction rate can be calculated by comparing the number of plaques formed in virus control group against that formed in the T and R groups. The average number of plaques of virus control group shall be in the range of 50-150.

The criteria for interpretation;

(1) Qualified: $T \geq (RA + RB)/2 - 0.33$

(2) Re-test: $(RA + RB)/2 - 0.66 < T < (RA + RB)/2 - 0.33$

(3) Unqualified: $T < (RA + RB)/2 - 0.66$

3.4.5 Thermostability test

Before release the final product shall be subject to thermostability test. The vaccine samples that have been exposed at 37°C for 7 days shall be titrated following Section 3.4.4. The vaccine that passes the thermostability test is considered satisfactory in potency test.

3.4.6 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be less than 50 ng/dose determined by ELISA.

3.4.7 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.4.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

3.4.9 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU per human dose (Appendix XIII E, the limit test of gel-clot method).

3.5 Test for sodium bisulfite solution

3.5.1 Content of sodium bisulfite

The concentration of sodium bisulfite shall be 1.5%-2.5% (Appendix VII E).

3.5.2 Validation test for nontoxicity

The test sample shall be diluted according to the concentration for usage. The test shall be performed by injecting i. p. each of two mice weighing 18-20 g with 0.5 ml of the diluted sample. Observe the inoculated mice for 7 days. All of them shall survive.

4 Storage, shipping and validity period

Store and ship at 2-8°C and protected from light. The validity period of the vaccine is 24 months starting from the date when the potency test proved qualified.

5 Package inserts

Directions for Use of Japanese Encephalitis Vaccine, Inactivated

[Drug name]

Adopted name: Japanese Encephalitis Vaccine,

Inactivated

[Constituents and characters]

Japanese encephalitis inactivated vaccine is a preparation of JE virus grown on monolayers of primary hamster kidney cells. After cultivation and harvest, the virus is inactivated to produce the vaccine. The vaccine is a clear, orange-red liquid, containing thimerosal as a preservative.

[Eligibles]

Healthy children from 6 months to 10 years of age and those including children and adults who intend to enter the endemic area from non-endemic areas.

[Function and use]

The product can induce immunity against JE virus in recipients following immunization. It is used to prevent Japanese encephalitis.

[Specifications]

2.0 ml, 5.0 ml or 10 ml per container. 0.5 ml per single human dose.

[Administration and dosage]

(1) In order to ease the pain of injection, add a quantity of sodium bisulfite solution into the vaccine container immediately prior to use. The vaccine is injectable while its colour changes from red to yellow.

(2) Inject s. c. the vaccine at deltoid insertion area of the lateral upper arm.

(3) For children of 6-12 months of age, the first and second shots are given at an interval of 7-10 days. The third shot shall be administered 6 months after the second shot; the fourth shot shall be given to the children of 4-10 years of age.

[Adverse reactions]

Generally there is no adverse reactions after inoculation. Some individuals may feel dizziness or have transient fever reaction, which normally does not last longer than 2 days and could be relieved spontaneously. Occasionally, sporadic skin rashes may appear and no particular treatment is needed. In case of necessity, symptomatic treatment might be adopted.

[Contraindications]

(1) The subjects with fever, acute or serious chronic disease, weakness.

(2) Those allergic to some drugs or food.

(3) Those with a history of convulsion.

[Precautions]

(1) Do not use the vaccine if any turbidity or colour change of content, foreign matters or leakage of container is found.

(2) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(3) Freezing of the vaccine is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Japanese Encephalitis Vaccine, Live

Japanese encephalitis (JE) live vaccine is a preparation of live attenuated JE virus (strain SA14-14-2) grown on the monolayers of primary hamster kidney cell cultures. After cultivation and harvest, the virus suspension is lyophilized to make the vaccine after the addition of a suitable stabilizer. It is used to prevent Japanese encephalitis.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for the production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Primary hamster kidney cells or those subcultured continuously for not more than five passages can be used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

Hamsters of 10-14 days of age are selected. Kidneys are extracted aseptically. The selected kidneys are minced with scissors and digested with trypsin solution. Dispersed cells are suspended in an appropriate culture medium; distribute them into culture bottles and then incubate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Inoculate the virus when confluent cell sheets are formed.

2.2 Virus seeds

2.2.1 Name and origin of virus strain

The attenuated JE virus (strain SA14-14-2) is used as the seed for the vaccine production.

2.2.2 Establishment of seed lot system

The Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of

Biologics shall apply.

The primary seed lot shall not exceed the 6th passage. The master seed lot shall not exceed the 8th passage. The working seed lot shall not exceed the 9th passage; and the final product shall not exceed the 10th passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Neutralization test is employed for the identification. Mix the virus from seed lot with the specific antibody against non-homologous JE virus. Keep the virus-antibody mixture at 37°C for 90 minutes. Inoculate the mixture onto the monolayers of hamster kidney cells or BHK₂₁ cells. Observe the results for 5-7 days after inoculation. The neurovirulence index shall be more than 1000.

2.2.3.2 Virus titration

The sample of virus seed shall be diluted 10-fold serially, of which at least three consecutive dilutions shall be inoculated respectively onto BHK₂₁ cells. Plaque titration method shall be used. The virus titers of freeze-dried seed lots shall be not less than $5.7 \lg \text{PFU/ml}$; for the liquid seed lots, not less than $7.2 \lg \text{PFU/ml}$.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix VIII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix VIII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix VIII C).

2.2.3.6 Stability of the gene sequence of E protein

The genetic stability shall be verified with the determination of the nucleotide sequence of protein E gene.

None of the changes is permitted in the coding sequence for following eight key amino acids on protein E, i.e. E-107 (Phenylalanine), E-138 (Lysine), E-176 (Valine), E-177 (Alanine), E-264 (Histidine), E-279 (Methionine), E-315 (Valine) and E-439 (Arginine).

The homology of the test nucleotide sequence of protein E gene to that of the attenuated strain of Japanese Encephalitis B virus (SA14-14-2, Gene Bank Registered number: D90195) shall be not less than 99.6%.

2.2.3.7 Test for immunogenicity

Prepare the original vaccine with the master seed virus and make 10-fold serial dilutions of which at least dilutions 10^{-3} , 10^{-4} and 10^{-5} of the vaccine are used to immunize mice weighing 10-12 g each;

ten mice for each dilution and each mouse receives s. c. 0.1 ml. Fourteen days after the immunization, challenge i. p. each mouse with 0.3 ml of the virulent JE virus (P_3 strain), in which the virus content shall be not less than 500 LD_{50} (i. p. titration). Meanwhile inoculate i. c. 0.03 ml of diluting medium into each mouse. Evaluate the results 14 days after the challenge. The ED_{50} value shall be not more than 3.0 lg PFU. The mortality of mice in the control group after challenge shall be not less than 80%.

2.2.3.8 Test for neurovirulence in monkeys

The test shall be carried out with the freeze-dried master seed of which the titer shall be not less than 5.7 lg PFU/ml. Make the seed virus 5-fold dilution (1 : 5). The diluted material shall be given to ten rhesus monkeys by inoculation of 0.5 ml into the thalamic region of each hemisphere and 0.2 ml into spinal cord, respectively. For the control group, two dilutions of the virulent strain (SA_{14}) are prepared, i. e. 10^2 PFU/ml and 10^3 PFU/ml with which four monkeys are injected respectively. The injections shall be given in the same way as that for the test group.

The ten monkeys in the test group of attenuated virus SA14-14-2 shall be observed for at least 18 days. No signs of illness shall occur. Only mild inflammatory reactions can be found at the injection sites of brain and spinal cord in histopathological examinations. However, in the control of virulent virus (SA_{14}), all the four monkeys in the group of 10^3 PFU/ml shall die within 8 days after inoculation; in the group of 10^2 PFU/ml at least two monkeys shall die. The predominant characteristics of histopathological changes in the monkeys of control group shall show necrosis of nerve cells, and inflammatory reactions shall be less intensive.

2.2.3.9 Test for neurovirulence in mice

Inject i. c. each of at least ten mice weighing 12-14 g with 0.03 ml of viral suspension of seed lot. Observe the mice for 14 days. The animals shall survive the observation period. Mice that die within 3 days after inoculation shall be excluded from final evaluation and for the test to be valid, no more than 20% of mice shall die within 3 days. The mice showing signs of illness 3 days after inoculation shall be killed, and their brains shall be extracted for testing the pathogenicity. The i. c. LD_{50} titer shall be not more than 3.0 lg $LD_{50}/0.03$ ml. Meanwhile, at least ten mice each weighing 10-12 g shall be inoculated s. c. with 0.1 ml of the 10% brain suspension. No mice shall show signs of JE virus infection during the 14 day observation period.

2.2.3.10 Neurotropism test in mice

Each of ten mice weighing 10-12 g shall be inoculated s. c. with 0.1 ml of the virus suspension of seed lot, and at the same time the right side of the mouse brain shall be pierced with a sterile needle. No

mice shall show signs of JE virus infection during the 14 day observation period.

2.2.3.11 Test for reversion of neurovirulence in suckling mice

Each of ten suckling mice of 3-5 days old shall be injected i. c. with 0.02 ml of the virus of working seed lot with a titer of not less than 7.2 lg PFU/ml (liquid virus seed) or not less than 5.7 lg PFU/ml (freeze-dried virus seed). Three of the suckling mice which manifest typical signs of illness shall be killed for testing the pathogenicity of their brains. The LD_{50} titer of the brain suspension shall be not more than 3.0 lg $LD_{50}/0.03$ ml tested i. c. in mice each weighing 12-14 g. Meanwhile at least ten mice each weighing 10-12 g shall be inoculated s. c. with 0.1 ml of the 10% brain suspension. No mice shall show signs of JE virus infection during the 14 day observation period.

2.2.4 Storage of virus seed lots

The seed lots shall be stored at or below -60°C .

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Earle solution containing lacto-albumin hydrolysate and a quantity of inactivated calf serum shall be used as the culture medium. Other suitable media can also be used. The calf serum used for cell cultivation shall be JE antibody negative. The quality of calf serum shall comply with the related requirements (Appendix III D).

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix III C).

2.3.4 Virus inoculation and cultivation

Cell cultures with a confluent monolayer shall be selected and washed thoroughly, to which a suitable volume of maintenance medium shall be added. Inoculate the seed virus to achieve 0.001 MOI (final titer in the maintenance medium being 2.7-3.7 lg PFU/ml), and keep the cell cultures for incubation at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

2.3.5 Virus harvest

About 72 hours after virus inoculation, harvest the virus suspension when CPE appears on the cell monolayer. Multiple harvests can be carried out after further cultivation with replacing maintenance media if the quality of cells is good enough.

2.3.6 Pooling of single virus harvests

Only the single virus harvest qualified in control tests shall be pooled. After clarification by filtration the pooled virus suspension is defined as the vaccine bulk.

2.3.7 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

The final bulk is prepared by pooling the qualified bulks, to which an amount of stabilizer is added. The amount of each final bulk shall not exceed 150 liters.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. During the filling process, the final bulk shall be kept cool in ice bath.

2.5.3 Specifications

0.5 ml or 2.5 ml of reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 5.4 lg PFU of live JE virus.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Virus titration

See Section 2.2.3.2. The titer of bulk shall be not less than 7.0 lg PFU/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.1.4 Test for reverse transcriptase activity

It complies with the test for reverse transcriptase (Appendix IX M). The result shall be negative.

3.2 Control tests on final bulk

3.2.1 Virus titration

See Section 2.2.3.2. The titer of the final bulk shall be not less than 6.8 lg PFU/ml.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile PBS shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

See Section 2.2.3.1.

3.3.2 Inspection on final containers

The product looks like a light yellow crisp cake. After reconstitution, it shall turn into a clear

liquid, orange-red or light-pink in colour, free of foreign matters.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Virus titration

See Section 2.2.3.2. The titer shall be not less than 5.7 lg PFU/ml.

3.3.5 Thermostability test

Before release the final product shall be subject to thermostability test which shall be performed at the same time with virus titration in parallel. The vaccine samples that have been exposed at 37°C for 7 days shall be titrated following Section 2.2.3.2. The virus titer shall be not less than 5.7 lg PFU/ml. The loss of virus titer of the heat exposed vaccine shall be not more than 1.0 lg PFU/ml.

3.3.6 Content of residual bovine serum albumin

The residual bovine serum albumin content shall be not more than 50 ng/dose determined by ELISA.

3.3.7 Test for safety

3.3.7.1 Test for neurovirulence in mice

See Section 2.2.3.9.

3.3.7.2 Test for reversion to neurovirulence in suckling mice

See Section 2.2.3.11.

3.3.8 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.4 Tests on diluent

The diluent is sterile PBS.

3.4.1 pH

The pH shall be 7.2-8.0 (Appendix V A).

3.4.2 Sterility test

It complies with the test for sterility (Appendix XII A).

4 Storage, shipping and validity period

Store and ship at or below 8°C, protected from light. The validity period of the vaccine is 18 months starting from the date when titration test proved qualified.

5 Package inserts

Directions for Use of Japanese Encephalitis Vaccine, Live

[Drug name]

Adopted name: Japanese Encephalitis Vaccine, Live

[Constituents and characters]

Japanese encephalitis live vaccine is a preparation of live attenuated JE virus (strain SA14-14-2) grown on the monolayer of hamster kidney cell cultures. After cultivation and harvest, an

appropriate stabilizer is added in the virus suspension, which is then lyophilized. The product looks like a light yellow crisp cake. After reconstitution, it shall turn into a clear, orange-red liquid.

[Eligibles]

Healthy children above 8 months of age and those including children and adults who intend to enter the endemic area from non-endemic area.

[Function and use]

The product can induce immunity against JE virus in recipients following immunization. It is used to prevent Japanese encephalitis.

[Specifications]

0.5 ml or 2.5 ml of reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 5.4 lg PFU of live JE virus.

[Administration and dosage]

(1) Reconstitute the freeze-dried vaccine with the accompanying vaccine diluent as stated on the label, and do not use the vaccine until it is reconstituted completely.

(2) Inject s.c. 0.5 ml of the vaccine at deltoid insertion area of the lateral upper arm.

(3) A portion of 0.5 ml of vaccine shall be given for a child at the age of 8 months, 2 years and 7 years respectively. No more inoculations are needed henceforth.

[Adverse reactions]

Transient fever may occur in a few recipients, which normally does not last longer than 2 days and could be relieved spontaneously. Occasionally, sporadic skin rashes may appear and commonly no particular treatment is needed. In case of necessity, symptomatic treatment might be helpful.

[Contraindications]

(1) Subjects with fever, acute infectious disease, otitis media, active tuberculosis, cardiac, renal or hepatic disease.

(2) Constitutional weakness, subjects with an allergic or epilepsy history.

(3) Subjects with congenital immunodeficiency, and those who are receiving or received immunodepressant therapy recently.

(4) Women in pregnancy.

[Precautions]

(1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.

(2) Do not use the vaccine if any leakage of container or clumps not dispersed on shaking are found, or the colour of the vaccine turned into red before reconstitution.

(3) The vaccine shall be used up within one hour after reconstitution at the ambient temperature of 2-8°C; discard the remaining vaccine afterwards, if any.

(4) Do not use the vaccine one month before or after administrating another live vaccine.

[Storage]

Store and ship at or below 8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Haemorrhagic Fever with Renal Syndrome (Type I) Vaccine, Inactivated

Haemorrhagic fever with renal syndrome type I vaccine is a preparation of Hantaan virus (HTNV) grown in primary cell cultures derived from gerbil kidney. After cultivation and harvest, the virus suspension is inactivated before adding aluminum hydroxide as an adjuvant to make the vaccine. It is used to prevent haemorrhagic fever with renal syndrome type I (HFRS type I).

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Primary cell cultures of gerbil kidney are used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

The kidneys of gerbils of 2 weeks old are extracted and minced with scissors aseptically. The tissue fragments are digested with trypsin solution. The cells are dispersed in a suitable medium such as MEM and inoculated into bottles for cultivation.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The strain Z₁₀ of HTNV shall be used as the seed for the production of HFRS type I vaccine.

2.2.2 Establishment of seed lot system

The Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics shall apply.

Primary seed and master seed lots shall be prepared using mice by i.c. inoculation. The primary seed lot shall not exceed the 12th passage. The master seed lot shall not exceed the 13th passage. Inoculate the master seed lot virus into primary cell cultures derived from gerbil kidney to prepare working seed lot. The working seed lot shall not exceed the 18th passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Mix the seed virus with a known immune serum against HTNV in equal volume. After incubation in 37°C water bath for 90 minutes inoculate the mixture onto the monolayer of Vero-E₆ cells. Observe the cells for 10-14 days. No CPE shall occur. The neutralization index determined by IFA shall be more than 1000.

2.2.3.2 Virus titration

The sample of working seed virus shall be diluted 10-fold serially to inoculate Vero-E₆ cells. The virus titer in Vero-E₆ cells determined by IFA shall be not less than 6.0 lg CCID₅₀/ml. The titer shall be not less than 7.0 lg CCID₅₀/ml by i.c. titration in suckling mice of 2-3 days or gerbils of 25-35 days of age.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix XIII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XIII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix XIII C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the seed virus of master seed lot to immunize i.m. four white rabbits weighing about 2 kg each in the hind leg. Each animal receives 1.0 ml twice at an interval of 7 days. Four weeks after the first injection, bleed the rabbits and separate the sera. The neutralizing titers of sera are determined by plaque reduction assay. 76-118 virus strain is used for challenge. Reference serum shall be tested in parallel as control. The neutralizing antibody titers in all the four rabbits of the test group shall be not less than 1:10.

2.2.4 Storage of virus seed lots

Virus seed lots shall be stored at or below -60°C.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

MEM or other suitable culture media containing a quantity of inactivated calf serum are used as the cell culture medium. The quality of calf serum shall comply with the related requirements in Appendix XIII D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix XIII C).

2.3.4 Virus inoculation and cultivation

Inoculate the virus into cell cultures with dense monolayers to make a final virus concentration of 4.0-5.0 lg CCID₅₀/ml. Incubate the containers at a suitable temperature for a certain period of time. Then discard the culture medium and flush the cell sheets with PBS to remove calf serum. Fresh maintenance medium shall be added to continue the cultivation.

2.3.5 Virus harvest

When the hemoagglutinin titer of virus suspension is equivalent to 1:64 or above, harvest the virus suspensions as well as cells. Pool the harvests followed by filtration and centrifugation.

2.3.6 Control tests on virus harvests

See Section 3.1.

2.3.7 Virus inactivation

β-propiolactone shall be added to the virus harvests in a proportion of 1:4000 and thimerosal is added to a final concentration of not more than 0.10 mg/ml. The inactivation process shall be carried out at 2-8°C for 7 days to inactivate the virus and hydrolyze the β-propiolactone.

2.3.8 Pooling

The inactivated virus harvests qualified in control tests shall be pooled for centrifugation and filtration. Vaccine bulk is made by adding a quantity of human blood albumin as a stabilizer to the filtrate.

2.3.9 Control tests on bulk

See Section 3.2.

2.4 Final bulk

2.4.1 Formulation

The final bulk is made by adding aluminum hydroxide as an adjuvant to a final concentration of not more than 0.70 mg/ml to the qualified bulk.

2.4.2 Control tests on final bulk

See Section 3.3.

2.5 Final product

2.5.1 Defining batches

The Requirements for Batches Defining of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of

Biologics shall apply.

2.5.3 Specifications

1.0 ml per container. 1.0 ml per single human dose.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on virus harvests

3.1.1 Virus titration

The sample shall be diluted 10-fold serially, of which at least three dilutions shall be inoculated onto the monolayers of Vero-E₆ cells or gerbil kidney cells. Evaluate the results by IFA after incubating the cells at a suitable temperature for around 7 days. The virus titer shall be not less than 6.0 lg CCID₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control tests on bulk

3.2.1 Validation test for effective inactivation

A portion of 0.1% of the total volume of the inactivated virus harvest suspension shall be sampled for the test. After dialysis the sample is inoculated onto Vero-E₆ cell cultures. Blind passages are conducted at intervals of 10-14 days. Three passages shall be carried out consecutively. Each passage shall be examined by IFA. All the results shall be negative.

3.2.2 Determination of antigen content

The titer of antigen shall be not less than 1 : 64 determined by reverse passive hemagglutination assay.

3.2.3 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.2.4 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.4 Control tests on final product

3.4.1 Identity test

See Section 2.2.3.6. The test shall be considered unsatisfactory if the potency test fails.

3.4.2 Inspection on final containers

The vaccine is a slightly turbid, orange-red liquid. After storage for a long time a precipitate may

form which can be dispersed on shaking. It shall be free of foreign matters.

3.4.3 Chemical tests

3.4.3.1 pH

The pH shall be 7.2-8.0 (Appendix V A).

3.4.3.2 Content of thimerosal

The thimerosal content shall be not more than 0.10 mg/ml (Appendix VII B).

3.4.3.3 Content of aluminum hydroxide

The content of aluminum hydroxide shall be not more than 0.70 mg/ml (Appendix VII F).

3.4.4 Potency test

See Section 2.2.3.6. The neutralizing antibody titer in each of four immunized rabbits shall be not less than 1 : 10.

3.4.5 Thermostability test

Before release, each lot of vaccine shall be subject to thermostability test. The test sample is exposed at 37°C for one week for the determination of potency (See Section 2.2.3.6). The test vaccine which passed the thermostability test is considered satisfactory in potency test.

3.4.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.4.7 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU per human dose (Appendix XII E, the limit test of gel-clot method).

3.4.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period of the vaccine is 18 months starting from the date when potency test proved qualified.

5 Package inserts

Directions for Use of Haemorrhagic Fever with Renal Syndrome (Type I) Vaccine, Inactivated

[Drug name]

Adopted name: Haemorrhagic Fever with Renal Syndrome (Type I) Vaccine, Inactivated

[Constituents and characters]

The product is a preparation of Hantaan virus grown in primary cell cultures derived from gerbil kidney. After cultivation and harvest, the vaccine is made by inactivation of the virus before adding aluminum hydroxide as an adjuvant. The vaccine is a slightly turbid, orange-red liquid, containing thimerosal as a preservative.

[Eligible]

Inhabitants living in the endemic area of HFRS and those who intend to enter the endemic area from non-endemic area. Those aged 10-60 years are the

high risk population.

[Function and use]

The product can induce immunity against Hantaan virus in recipients following immunization. It is used to prevent HFRS (type I).

[Specifications]

1.0 ml per container. 1.0 ml per single human dose.

[Administration and dosage]

(1) Inject i. m. in the deltoid muscle of the lateral upper arm.

(2) For primary immunization, three injections are given on days 0, 7 and 28, respectively. The booster injection shall be given one year after the primary immunization. One ml of the vaccine shall be given for each injection.

[Adverse reactions]

Generally there are no adverse reactions after inoculation. Care shall be taken for a few recipients with fever, skin rashes or dizziness after injection. Appropriate treatment could be given in case of need. Because the vaccine contains aluminum hydroxide adjuvant, induration, mild swelling or pain may appear at the injection site after inoculation in a few recipients. Normally these reactions could be relieved spontaneously within 1-3 days.

[Contraindications]

(1) Subjects with fever, acute or serious chronic disease, nervous system diseases.

(2) Those with history of anaphylaxis, or with a history of allergic reaction to antibiotics and/or biologics.

(3) Women during lactation or pregnancy.

[Precautions]

(1) Shake the container before use.

(2) Do not use the vaccine if any leakage of container, abnormal turbidity of the content, changed colour of the content, foreign matters or clumps not dispersed on shaking are found.

(3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(4) Freezing of the vaccine is contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;

Address;

Zip code;

Telephone number;

Fax;

Web site;

Haemorrhagic Fever with Renal Syndrome (Type II) Vaccine, Inactivated

Haemorrhagic fever with renal syndrome (type II) vaccine is a preparation of Seoul virus (SEOV) grown in primary cell cultures derived from hamster kidney. After cultivation and harvest, the virus suspension is inactivated before adding aluminum hydroxide as an adjuvant to make the vaccine. It is used to prevent haemorrhagic fever with renal syndrome type II (HFRS type II).

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Primary cell cultures derived from hamster kidney are used for the vaccine production.

2.1.1 Management and control tests on cell substrate

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

The kidneys of golden hamsters of about 2 weeks old are extracted and minced with scissors aseptically. The tissue fragments are digested with trypsin solution. The cells are dispersed in a suitable medium and distributed into bottles for cultivation at 37°C.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The strain L₉₉ of SEOV is used as the seed for the vaccine production.

2.2.2 Establishment of seed lot system

The Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics shall apply. Master seed lot shall be prepared by serial passage in primary hamster kidney cell cultures. The primary seed lot shall be the 13th passage. The master seed shall not exceed the 16th passage. The working seed lot shall not exceed the 20th passage.

2.2.3 Control tests on virus seed lots

For master seed lot, control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Mix the seed virus with known immune serum against SEOV in equal volume. After incubation in 37°C water bath for 90 minutes, inoculate the mixture onto monolayers of Vero-E₆ cells. Observe the cells for 10-14 days. The neutralization index determined by IFA shall be more than 1000.

2.2.3.2 Virus titration

Primary hamster kidney cells are used in the titration for infectivity of the seed virus with IFA technique. The titer shall be not less than 7.0 lg ID₅₀/ml.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix X A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix X B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix X C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the seed virus from master seed lot to immunize i. m. four white rabbits weighing about 2 kg each in hind leg. Each animal receives 1.0 ml twice at an interval of 14 days. Four weeks after the first injection, bleed the rabbits and separate the sera. The neutralizing titers of sera are determined by plaque reduction assay. UR virus strain is used for challenge. Reference serum shall be tested in parallel as control. The neutralizing antibody titers in all the four rabbits of the test group shall be not less than 1 : 10.

2.2.4 Storage of virus seed lots

Virus seed lots shall be stored at or below -60°C. Liquid working seed lots shall be stored for not more than one year.

2.3 Bulk**2.3.1 Cell substrate preparation**

See Section 2.1.2.

2.3.2 Culture medium

Earle solution containing lacto-albumin hydrolysate and a quantity of inactivated calf serum shall be used as the culture medium. Other suitable culture media can also be used. The quality of calf serum shall comply with the related requirements in Appendix X D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix X C).

2.3.4 Virus inoculation and cultivation

Cell cultures with confluent monolayer are selected. Inoculate a quantity of seed virus. After incubation at a suitable temperature for a period of time, the growth medium shall be replaced by fresh maintenance medium to continue the

cultivation.

2.3.5 Virus harvest

After cultivation for a certain period of time, collect the culture fluid, i. e. virus harvest.

2.3.6 Control tests on virus harvest

See Section 3.1.

2.3.7 Virus inactivation

Formalin is added to the virus harvests in a proportion of 1:4000. The inactivation process shall be conducted at an appropriate temperature for a certain period of time.

2.3.8 Pooling

The inactivated harvests qualified in control tests shall be pooled. After centrifugation, thimerosal shall be added to the pooled harvests as a preservative to make a final concentration of not more than 0.10 mg/ml. It is defined as the bulk.

2.3.9 Control tests on bulk

See Section 3.2.

2.4 Final bulk**2.4.1 Formulation**

The final bulk is made by adding aluminum hydroxide as an adjuvant to a final concentration of not more than 0.70 mg/ml to the qualified bulk.

2.4.2 Control tests on final bulk

See Section 3.3.

2.5 Final product**2.5.1 Defining batches**

The Requirements for Batches Defining of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1.0 ml per container, 1.0 ml per single human dose.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests**3.1 Control tests on virus harvest****3.1.1 Virus titration**

The sample shall be diluted 10-fold serially, of which at least three dilutions shall be inoculated onto primary hamster kidney cells or other suitable cells. Evaluate the results using IFA after incubating the cells at a suitable temperature for 10-12 days. The virus titer shall be not less than 6.5 lg CCID₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix X A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas

(Appendix VII B).

3.2 Control tests on bulk

3.2.1 Validation test for effective inactivation

A portion of 0.1% of the total volume of the inactivated harvest suspension shall be sampled for the test. After dialysis the sample is inoculated onto Vero-E₆ cell cultures. Blind passages are conducted at intervals of 10-14 days. Three passages shall be carried out consecutively. Each passage shall be examined by IFA. All the results shall be negative.

3.2.2 Determination of antigen content

The titer of antigen in the bulk shall be not less than 1 : 64 determined by ELISA.

3.2.3 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.2.4 Sterility test

It complies with the test for sterility (Appendix VII A).

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix VII A).

3.4 Control tests on final product

3.4.1 Identity test

See Section 2.2.3.6. The test shall be considered unsatisfactory if the potency test fails.

3.4.2 Inspection on final containers

The vaccine is a turbid, orange-red liquid. After storage for a long time, a precipitate may form which can be dispersed on shaking. It shall be free of foreign matters.

3.4.3 Chemical tests

3.4.3.1 pH

The pH shall be 7.2-8.0 (Appendix V A).

3.4.3.2 Content of thimerosal

The thimerosal content shall be not more than 0.10 mg/ml (Appendix VII B).

3.4.3.3 Content of aluminum hydroxide

The content of aluminum hydroxide shall be not more than 0.70 mg/ml (Appendix VII F).

3.4.3.4 Content of free formaldehyde

The content of free formaldehyde shall be not more than 0.50 mg/ml (Appendix VI L).

3.4.4 Potency test

See Section 2.2.3.6. The neutralizing antibody titer in each of the four immunized rabbits shall be not less than 1 : 10.

3.4.5 Thermostability test

Before release each lot of vaccine shall be subject to thermostability test. The test sample is exposed at 37°C for one week for the determination of potency (See Section 2.2.3.6). The test vaccine which passed the thermostability test is considered

satisfactory in potency test.

3.4.6 Sterility test

It complies with the test for sterility (Appendix VII A).

3.4.7 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU per human dose (Appendix VII E, the limit test of gel-clot method).

3.4.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period of the vaccine is 18 months starting from the date when the potency test proved qualified.

5 Package inserts

Directions for Use of Haemorrhagic Fever with Renal Syndrome (Type II) Vaccine, Inactivated

[Drug name]

Adopted name: Haemorrhagic Fever with Renal Syndrome (Type II) Vaccine, Inactivated

[Constituents and characters]

The vaccine is a preparation of Seoul virus grown in primary cell cultures derived from hamster kidney. After cultivation and harvest, the vaccine is made by inactivation of the virus before adding aluminum hydroxide as an adjuvant. The vaccine is a slightly turbid, orange-red liquid, containing thimerosal as a preservative.

[Eligible]

Inhabitants living in the endemic area of HFRS and those who intend to enter the endemic area from non-endemic area. Those aged 16-60 years are the high risk population.

[Function and use]

The product can induce immunity against Seoul virus in recipients following immunization. It is used to prevent HFRS (Type II).

[Specification]

1.0 ml per container. 1.0 ml per single human dose.

[Administration and dosage]

(1) Inject i. m. in the deltoid muscle of the lateral upper arm.

(2) For primary immunization, three injections should be given on days 0, 14 and 28, respectively. The booster injection shall be given one year after the primary immunization. One ml of the vaccine shall be given for each injection.

[Adverse reactions]

Care shall be taken for a few recipients with fever, skin rashes or dizziness after injection. Appropriate treatment could be given in case of need. Because the vaccine contains aluminum hydroxide

adjuvant, induration, mild swelling or pain may appear at the injection site after inoculation in a few recipients. Normally these reactions could be relieved spontaneously within 1-3 days.

[Contraindications]

- (1) Subjects with fever, acute or serious chronic disease, nervous system diseases.
- (2) Those with history of anaphylaxis, or with a history of allergic reaction to antibiotics and/or biologics.
- (3) Women during lactation or pregnancy.

[Precautions]

- (1) Shake the container before use.
- (2) Do not use the vaccine if any leakage of container, abnormal turbidity of the content, changed colour of the content, foreign matters or clumps not dispersed on shaking are found.
- (3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.
- (4) Freezing of the vaccine is contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;
Address;
Zip code;
Tel;
Fax;
Web site;

Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine, Inactivated

Haemorrhagic fever with renal syndrome bivalent vaccine is a preparation made from Hantaan virus (HFRS virus type I) and Seoul virus (HFRS virus type II) grown separately in primary cell cultures derived from gerbil kidney. After cultivation and harvest, the virus suspensions of two types are pooled and inactivated before adding aluminum hydroxide as an adjuvant to make the vaccine. It is used to prevent haemorrhagic fever with renal syndrome (HFRS, both types I and II).

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the require-

ments set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Primary cell cultures derived from gerbil kidney are used for the vaccine production.

2.1.1 Management and control tests on cell substrate

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

The kidneys of gerbil of 10-20 days old are extracted and minced with scissors aseptically. The tissue fragments are digested with trypsin solution. The cells are dispersed in MEM or other appropriate media and distributed into bottles for cultivation.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Hantaan virus strain Z₁₀ and Seoul virus strain Z₃₇ are used as the seeds for the vaccine production.

2.2.2 Establishment of seed lot system

The Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics shall apply.

Mouse brains infected with the Z₁₀ strain virus or with the Z₃₇ strain virus are used to make the primary seed lot and master seed lot. The working seed lot is prepared by infection of primary gerbil kidney cell cultures with the master seed. The primary seed lot (strain Z₁₀) for type I shall not exceed the 12th passage; the master seed lot shall not exceed the 13th passage and the working seed lot shall not exceed the 18th passage. For type II (strain Z₃₇), the primary seed lot shall not exceed the 10th passage; the master seed lot shall not exceed the 11th passage, and the working seed lot shall not exceed the 16th passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Mix the seed virus of two strains separately with the corresponding type specific immune serum in equal volume. After incubation in 37°C water bath for 90 minutes, inoculate the two kinds of mixtures onto monolayer cultures of Vero-E₆ cells, respectively. Observe the cells for 10-14 days. No CPE shall occur. Both the neutralization indexes determined by IFA for the two types of viruses shall be higher than 1000.

2.2.3.2 Virus titration

The sample of seed virus shall be diluted 10-fold serially to inoculate Vero-E₆ cell cultures. The virus titers in Vero-E₆ cell cultures determined by

IFA shall be not less than 6.0 lg CCID₅₀/ml. The titer shall be not less than 7.0 lg CCID₅₀/ml by i.c. titration in suckling mice of 2-3 days or gerbils of 25-30 days of age.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix X A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix X B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix X C).

2.2.3.6 Test for immunogenicity

Prepare the original bivalent vaccine with the master seed virus to immunize i.m. four white rabbits weighing about 2 kg each in hind leg. Each animal receives 1.0 ml twice at an interval of 7 days. Four weeks after the first injection, bleed the rabbits and separate the sera. The neutralizing titers of sera are determined by plaque reduction assay. The viruses of strain 76-118 or strain UR are used for challenge, respectively. Reference sera shall be tested in parallel as control. Both the neutralizing antibody titers of any immunized rabbit for type I and type II shall be not less than 1 : 10.

2.2.4 Storage of virus seeds

Virus seed lots shall be stored at or below -60°C. The storage period of liquid working seed lots stored at or below -60°C shall not exceed one year.

2.3 Monovalent bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

MEM or other suitable culture media containing a quantity of inactivated calf serum are used as the cell culture medium. The quality of calf serum shall comply with the related requirements in Appendix X D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix X C).

2.3.4 Virus inoculation and cultivation

Cell cultures with confluent monolayers are selected. Inoculate the seed viruses (strain Z₁₀, type I and strain Z₃₇, type II), respectively. After incubation at suitable temperature for a period of time, discard the growth medium and flush cell sheet with PBS to remove the calf serum. Add a quantity of fresh maintenance medium to continue the cultivation for 3-5 days.

2.3.5 Virus harvest

Before harvest the cells shall be examined by IFA and the infection rate shall be not less than 95%.

Collect the virus suspensions, i.e. the monovalent virus harvest.

2.3.6 Control tests on monovalent virus harvest

See Section 3.1.

2.3.7 Virus inactivation

β-propiolactone shall be added to the virus harvests in a proportion of 1 : 4000 and thimerosal is added to a final concentration of not more than 0.10 mg/ml. The inactivation process shall be conducted at 2-8°C for 7 days or at 2-8°C overnight followed by 37°C for 2 hours to inactivate virus and hydrolyze β-propiolactone.

2.3.8 Pooling

The inactivated virus harvests qualified in control tests shall be pooled for centrifugation and filtration. The monovalent bulk is made by adding a quantity of human albumin as a stabilizer to the filtrate.

2.3.9 Control tests on monovalent bulk

See Section 3.2.

2.4 Final bulk

2.4.1 Formulation

Mix the monovalent bulks of two types qualified in control tests in equal volume to make a bivalent bulk mixture. The final bulk of the bivalent vaccine is made by adding aluminum hydroxide as an adjuvant to a final concentration of not more than 0.70 mg/ml in the mixture.

2.4.2 Control tests on final bulk

See Section 3.3.

2.5 Final product

2.5.1 Batch defining

The Requirements for Batches Defining of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1.0 ml per container. 1.0 ml per single human dose.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on monovalent virus harvest

3.1.1 Virus titration

The sample shall be diluted 10-fold serially, of which at least three dilutions shall be inoculated onto monolayers of Vero-E₆ cells or primary gerbil kidney cells. Evaluate the results using IFA after incubating the cells at an appropriate temperature for around 7 days. The virus titer shall be not less than 6.0 lg CCID₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix



III A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

3.2 Control tests on monovalent bulk

3.2.1 Validation test for effective inactivation

A portion of 0.1% of the total volume of each bulk shall be sampled for the test. After dialysis the sample is inoculated onto Vero-E₆ cell cultures. Blind passages are conducted at intervals of 10-14 days. Three passages shall be carried out consecutively. Each passage shall be examined by IFA. All the results shall be negative.

3.2.2 Sterility test

It complies with the test for sterility (Appendix III A).

3.2.3 Determination of antigen content

The titer of antigen shall be not less than 1:64 determined by reverse passive hemagglutination assay.

3.2.4 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix III A).

3.4 Control tests on final product

3.4.1 Identity test

See Section 2.2.3.6. The test shall be considered unsatisfactory if the potency test fails.

3.4.2 Inspection on final containers

The vaccine is a slightly turbid, orange-red liquid. After storage for a long time, a precipitate may form which can be dispersed on shaking. It shall be free of foreign matters.

3.4.3 Chemical tests

3.4.3.1 pH

The pH shall be 7.0-8.0 (Appendix V A).

3.4.3.2 Content of thimerosal

The thimerosal content shall be not more than 0.10 mg/ml (Appendix VII B).

3.4.3.3 Content of aluminum hydroxide

The content of aluminum hydroxide shall be not more than 0.70 mg/ml (Appendix VII F).

3.4.4 Potency test

See Section 2.2.3.6. The neutralizing antibody titer in each of the four immunized rabbits shall be not less than 1:10 for both type I and type II.

3.4.5 Thermostability test

Before release each lot of vaccine shall be subject to thermostability test. The test sample is exposed at 37°C for one week for the determination of potency (See Section 2.2.3.6). The test vaccine which

passed the thermostability test is considered satisfactory in potency test.

3.4.6 Sterility test

It complies with the test for sterility (Appendix III A).

3.4.7 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU per human dose (Appendix III E, the limit test of gel-clot method).

3.4.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix III F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light.

The validity period is 18 months starting from the date when potency test proved qualified.

5 Package inserts

Directions for Use of Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine, Inactivated

[Drug name]

Adopted name: Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine, Inactivated

[Constituents and characters]

The vaccine is a preparation of HNTV and Seoul virus grown in primary cell cultures derived from gerbil kidney separately. After cultivation and harvest, the vaccine is made by inactivation of the virus before adding aluminum hydroxide as an adjuvant. The vaccine is a slightly turbid, orange-red liquid, containing thimerosal as a preservative.

[Eligible]

Inhabitants living in the endemic area of HFRS and those who intend to enter the endemic area from non-endemic area. Those aged 16-60 years are the high risk population.

[Function and use]

The product can induce immunity against HFRS virus types I and II in recipients following immunization. It is used to prevent HFRS (types I and II).

[Specifications]

1.0 ml per container. 1.0 ml per single human dose.

[Administration and dosage]

(1) Inject i. m. in the deltoid muscle of the lateral upper arm.

(2) For primary immunization, two injections are given on days 0, and 14, respectively. One booster dose of 1 ml shall be given 6 months after the primary immunization. One ml of the vaccine shall be given for each injection.

[Adverse reactions]

Generally there are no adverse reactions after inoculation. Care shall be taken for a few recipients with fever, skin rashes or dizziness after injection. Appropriate treatment could be given in

case of need. Because the vaccine contains aluminum hydroxide adjuvant, induration, mild swelling or pain may appear at the injection site after inoculation in a few recipients. Normally these reactions could be relieved spontaneously within 1-3 days.

[Contraindications]

- (1) Subjects with fever, acute or serious chronic disease, nervous system diseases.
- (2) Those with history of anaphylaxis, or with a history of allergic reaction to antibiotics and/or biologics.
- (3) Women during lactation or pregnancy.

[Precautions]

- (1) Shake the container before use.
- (2) Do not use the vaccine if any leakage of container, abnormal turbidity of the content, changed colour of the content, foreign matters or clumps not dispersed on shaking are found.
- (3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.
- (4) Freezing of the vaccine is contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:
Address:
Zip code:
Tel:
Fax:
Web site:

Rabies Vaccine (Vero Cell) for Human Use

The rabies vaccine is a liquid preparation of rabies fixed virus grown in Vero cells. After cultivation and harvest, the virus suspension is inactivated, concentrated and purified, to which a suitable stabilizer is then added. Aluminum hydroxide may be added as an adjuvant. It is used to prevent rabies in human.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for production

Vero cells shall be used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animals Cell Substrates Used for Production of Biologics shall apply. The passage number of cell seed from cell banks used in the vaccine production shall not exceed the approved limit.

2.1.2 Cell substrate preparation

Cells from a single or several ampoules of the working cell bank are resurrected and expanded by serial subcultures up to a quantity enough for making one batch of vaccine. The quantity of the cells can be defined as a cell lot. During the subcultures of the cells, trypsin or other suitable digestive solutions at an appropriate concentration are added onto the monolayer cell cultures to disperse cells. The dispersed cells are homogenized with suitable growth medium and distributed into culture bottles. Incubate the cells at 37°C to form monolayers.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Strain CTN-1V and strain aGV or other rabies fixed virus strains adapted in Vero cells can be used as the seed for the vaccine production.

2.2.2 Establishment of virus seed lot

It complies with the Requirements for Bacterial and Viral Strains Used for Manufacture and Quality Control of Biologics. The maximum passage number of the seed lots shall not exceed the approved limits.

2.2.3 Control tests on virus seed lots

For master seed lots, comprehensive control tests described below shall be carried out; for working seed lots, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Neutralization test by i.c. route in mice shall be carried out to identify the specificity of the seed. The neutralization index shall be not less than 500.

2.2.3.2 Virus titration

Inoculate the virus suspension of each dilution i.c. in at least six mice each weighing 11-13 g. The titer shall be not less than 7.5 lg LD₅₀/ml.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix XII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix XII C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the seed virus of master seed lot to immunize i. p. a group of mice each weighing 12-14 g. Each animal receives 0.5 ml twice at an interval of one week. Two weeks after the first inoculation, challenge intracerebrally the immunized mice individually with 0.03 ml of CVS virus diluted 10-fold serially and ten mice are required for each dilution. The CVS virus shall be titrated in parallel in mice with the same weight as a control, ten mice are required for each dilution. The protective index of the virus seed shall be not less than 100.

2.2.4 Storage of virus seed lots

The virus seed lots shall be stored at or below -60°C , the liquid seed lots shall be stored for not more than 2 years.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

MEM, medium 199 or other suitable media containing a quantity of inactivated calf serum can be used as culture medium. The quality of calf serum shall comply with the related requirements (Appendix III D).

2.3.3 Tests for adventitious viruses on control cells

It complies with the tests for adventitious viruses (Appendix III C).

2.3.4 Virus inoculation and cultivation

Inoculate the virus seed at 0.01-0.1 MOI or to a final concentration of 4.5-5.5 lg LD₅₀/ml onto well grown cell cultures. Incubate the cultures at a suitable temperature for a certain period of time. Discard the culture medium and flush the cell sheets with PBS to remove calf serum. A quantity of maintenance medium is added to continue the cultivation at 33-35°C.

2.3.5 Virus harvest

After cultivation for a period of time, harvest the virus suspension; it is defined as a single virus harvest. A number of harvests can be achieved depending on the situation of cell growth.

2.3.6 Control tests on single viral harvest

See Section 3.1.

2.3.7 Virus inactivation

β -propiolactone shall be added to the virus harvests in a proportion of 1 : 4000. Inactivation shall be carried out at a suitable temperature for a period of time.

2.3.8 Pooling, concentration and purification

2.3.8.1 Pooling and concentration

A number of single harvests derived from the same lot of cell cultures can be pooled into one batch following strictly aseptic procedures. The yield shall be properly concentrated by means of ultra-

filtration or other suitable approaches.

2.3.8.2 Purification

The concentrated virus suspension qualified in control tests shall be purified by means of chromatography or other appropriate methods. A suitable amount of human albumin can be added as a stabilizer and thimerosal added as a preservative to make the bulk.

2.3.9 Control tests on bulk

See Section 3.2.

2.4 Final bulk

2.4.1 Formulation

Formulation shall be carried out according to its protein and antigen contents. The protein content of each single human dose shall be less than 120 μg . Aluminum hydroxide may be added as an adjuvant to prepare the final bulk; its final concentration shall be not more than 0.70 mg/ml.

2.4.2 Control tests on final bulk

See Section 3.3.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1.0 ml per container, 1.0 ml per single human dose. The potency of the vaccine shall be not less than 2.5 IU.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on single virus harvest

3.1.1 Virus titration

See Section 2.2.3.2. The titer shall be not less than 6.0 lg LD₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix III A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

3.2 Control tests on bulk

3.2.1 Determination of antigen content

A suitable method shall be adopted for determination of antigen content.

3.2.2 Validation test for effective inactivation

The test sample shall be inoculated i. c. into twenty mice each weighing 11-13 g, 0.03 ml for each. Observe the animals for 14 days. All of them shall survive the observation period. Those that die

within 3 days after injection shall be excluded from the final evaluation.

3.2.3 Protein content

Sample shall be collected from purified preparation prior to adding human blood albumin. The content shall be not more than 120 µg/dose (Appendix VI B, method 2).

3.2.4 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.2.5 Determination of residual DNA content of Vero cells

The residual DNA content of Vero cells shall be not more than 100 pg/dose (Appendix IX B).

3.2.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.4 Control tests on final product

3.4.1 Identity test

See Section 3.4.4. The test shall be considered unsatisfactory if the potency test fails.

3.4.2 Inspection on final containers

The vaccine is a turbid, milky-white liquid. After storage for a long time, a precipitate may form which can be dispersed on shaking. It should be free of foreign matters.

3.4.3 Chemical tests

3.4.3.1 pH

The pH shall be 7.2-8.0 (Appendix V A).

3.4.3.2 Content of thimerosal

The content of thimerosal shall be not more than 0.10 mg/ml (Appendix VII B).

3.4.3.3 Content of aluminum hydroxide

The vaccine with adjuvant shall be subject to this test. The content of aluminum hydroxide shall be not more than 0.70 mg/ml (Appendix VII F).

3.4.4 Potency test

The potency of the vaccine shall be not less than 2.5 IU/dose (Appendix XI A).

3.4.5 Thermostability test

Before release each lot of vaccine shall be subject to thermostability test. The test sample is exposed at 37°C for 14 days for the determination of potency (See Section 3.4.4). The test vaccine which passed the thermostability test is considered satisfactory in potency test.

3.4.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.4.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.4.8 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU/dose (Appendix XII E, the limit test of gel-clot method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 12 months starting from the date when potency test proved qualified.

5 Package inserts

Directions for Use of Rabies Vaccine (Vero Cell) for Human Use

[Drug Name]

Adopted name: Rabies Vaccine (Vero Cell) for Human Use

[Constituents and characters]

The vaccine is a liquid preparation of rabies fixed virus grown in Vero cells. After cultivation and harvest, the virus suspension is inactivated, concentrated, purified, to which a suitable stabilizer is then added. Aluminum hydroxide may be added as an adjuvant. It is a turbid, milky-white liquid, containing thimerosal as a preservative.

[Eligibles]

If a person is bitten or scratched by a rabid dog or other rabid animals, regardless of age or sex, the wounds shall be cleaned immediately (flush the wounds repeatedly with clean water or soap water, followed by applying iodine tincture or ethanol for several times), and the exposed person shall be inoculated with the vaccine according to the post-exposure schedule as soon as possible. The persons at risk of contacting rabies virus (such as veterinarians, animal breeders, forestry workers, workers in slaughterhouse and staffs in rabies laboratory) shall be immunized following the pre-exposure treatment schedule.

[Function and use]

The preparation can induce immunity against rabies virus in recipients following immunization. It is used to prevent rabies in human.

[Specifications]

1.0 ml per container. 1.0 ml per single human dose. The potency of the vaccine shall be not less than 2.5 IU.

[Administration and dosage]

(1) The vaccine shall be shaken homogeneously before use.

(2) The deltoid muscle of the upper arm is the recommended site for i.m. administration. For young children, inoculate the vaccine in the muscle at anterolateral aspect of the thigh.

(3) Post-exposure schedule for immunization: Normally one dose of the vaccine shall be administered to the exposed person on days 0 (the first day or the intraday), 3 (the fourth day,

analogically henceforth), 7, 14, and 28, consecutively; five doses in total. Children shall be treated in the same way. It is recommended to double the first dose of vaccine in case of one of the following situations:

- ① The exposed person was injected with immunoglobulin or antiserum one month before the day of receiving rabies vaccine.
- ② Those with congenital or acquired immunodeficiency.
- ③ Those receiving immunosuppressant (including antimalaria drug).
- ④ The elderly or patients with chronic diseases;
- ⑤ Administration of rabies vaccine becomes available to the exposed persons 48 hours or longer after exposure.

Post-exposure treatment shall be dependent on the following classification of wound severity:

Category I: those petting animal, licked by animal on intact skins without any breaks—neither wound treatment nor administration of vaccine is necessary.

Category II: those bitten or scratched by animal on skins but without bleeding; or licked on skins with breaks—vaccine shall be administered following the post-exposure immunization schedule.

Category III: those with single or multiple biting wounds on skins with bleeding or scratched with bleeding; mucous membrane was contaminated by saliva of suspected or confirmed rabid animal—the exposed person shall be treated immediately with rabies vaccine, rabies antiserum (40 IU/kg, horse origin) or immunoglobulin (20 IU/kg, human origin). If anatomically feasible, infiltration injection of the remaining serum (horse or human origin) shall be performed as much as possible around the wound (s); the rest, if any, shall be injected intramuscularly.

(4) Preexposure immunization schedule: A total of three shots given on days 0, 7 and 28.

(5) Recommendation of boosters for those immunized with rabies vaccine previously:

- ① Complete post-exposure immunization course was conducted in the recent one year; if bitten by a suspected rabid animal, one dose given on days 0 and 3 separately.
- ② Complete post-exposure immunization course was conducted in the previous year; if bitten by a suspected rabid animal, carry out a complete immunization course again.
- ③ Complete post-exposure immunization course was conducted in the last 3 years, and followed by boosters; if bitten by a suspected rabid animal, one dose of vaccine shall be given on days 0 and 3 separately.
- ④ Complete post-exposure immunization course and booster (s) was conducted more than 3 years ago; if bitten by a suspected rabid animal, a complete post-exposure immunization course shall be executed.

[Adverse reactions]

After inoculation, mild local or systemic reactions may occur, which could be relieved spontaneously. Occasionally rashes may appear. In case of some serious adverse reactions, such as immediate anaphylactic reactions, angioneurotic edema or urticaria, symptomatic treatment is recommended.

[Contraindications]

(1) Because rabies is a fatal disease, there are no contraindications for post-exposure immunization.

(2) For preexposure immunization, it is not recommended to immunize eligible individuals with fever, acute disease, serious chronic disease, nervous system disease, and with a history of allergic reaction to antibiotics and/or biological products. It is recommended to postpone the administration of the vaccine for women in pregnancy or in lactation, if feasible.

[Precautions]

(1) Do not use the vaccine if the vaccine contains any foreign substance, or any leakage of container or illegible label is found.

(2) Alcoholic drinks, strong tea, pungent food and strenuous exercise shall be avoided after injection of the vaccine.

(3) Do not inject the vaccine in the gluteal region.

(4) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

12 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Rabies Vaccine (Vero Cell) for Human Use, Freeze-dried

The rabies vaccine is a freeze-dried preparation of rabies fixed virus grown in Vero cells. After cultivation and harvest, the virus suspension is inactivated, concentrated and purified, and lyophilized after an addition of a suitable stabilizer. It is used to prevent rabies in human.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrate for production

Vero cells shall be used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animals Cell Substrates Used for Production of Biologics shall apply. The maximum passage number of cell seeds from cell banks used in the vaccine production shall not exceed the approved limit.

2.1.2 Cell substrate preparation

Cells from a single or several ampoules of the working cell bank are resurrected and expanded by serial subcultures up to a quantity enough for making one batch of vaccine. The quantity of the cells can be defined as a cell lot. During the subcultures of the cells, trypsin or other suitable digestive solutions at an appropriate concentration are added onto the monolayer cell cultures to disperse cells. The dispersed cells are homogenized with suitable growth medium and distributed into culture bottles. Incubate the cells at 37°C to form monolayers.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Strain CTN-1V and strain aGV or other rabies fixed virus strains adapted in Vero cells can be used as the seed for the vaccine production.

2.2.2 Establishment of virus seed lot

It complies with the Requirements for Bacterial and Viral Strains Used for Manufacture and Quality Control of Biologics. The maximum passage number of the seed lots shall not exceed the approved limits.

2.2.3 Control tests on virus seed lots

For master seed lots, comprehensive control tests described below shall be carried out; for working seed lots, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Neutralization test by i. c. route in mice shall be carried out to identify the specificity of the seed. The neutralization index shall be not less than 500.

2.2.3.2 Virus titration

Inoculate the virus suspension of each dilution i. c. into at least six mice each weighing 11-13 g. The titer shall be not less than 7.5 lg LD₅₀/ml.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix VIII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix VIII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses

(Appendix VIII C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the seed virus of master seed lot to immunize i. p. a group of mice each weighing 12-14 g. Each animal receives 0.5 ml twice at an interval of one week. Two weeks after the first inoculation, challenge intracerebrally the immunized mice individually with 0.03 ml of CVS virus diluted 10-fold serially and ten mice are required for each dilution. The CVS virus shall be titrated in parallel in mice with the same weight as a control, ten mice are required for each dilution. The protective index of the virus seed shall be not less than 100.

2.2.4 Storage of virus seed lots

The virus seed lots shall be stored at or below -60°C, the liquid seed lots shall be stored for not more than 2 years.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

MEM, medium 199 or other suitable media containing a quantity of inactivated calf serum can be used for culture medium. The quality of calf serum shall comply with the related requirements (Appendix VIII D).

2.3.3 Tests for adventitious viruses on control cells

It complies with the tests for adventitious viruses (Appendix VIII C).

2.3.4 Virus inoculation and cultivation

Inoculate the virus seed at 0.01-0.1 MOI or to a final concentration of 4.5-5.5 lg LD₅₀/ml onto well grown cell cultures. Incubate the cultures at a suitable temperature for a certain period of time. Discard the culture medium and flush the cell sheets with PBS to remove calf serum. A quantity of maintenance medium is added to continue the cultivation at 33-35°C.

2.3.5 Virus harvest

After cultivation for a period of time, harvest the virus suspension; it is defined as a single virus harvest. A number of harvests can be achieved depending on the situation of cell growth.

2.3.6 Control tests on single viral harvest

See Section 3.1.

2.3.7 Virus inactivation

β-propiolactone shall be added to the virus harvests in a proportion of 1 : 4000. Inactivation shall be carried out at a suitable temperature for a period of time.

2.3.8 Pooling, concentration and purification

2.3.8.1 Pooling and concentration by ultrafiltration

A number of single harvests derived from the same lot of cell cultures can be pooled into one batch

following strictly aseptic procedures. The yield shall be properly concentrated by means of ultra-filtration or other suitable approaches.

2.3.8.2 Purification

The concentrated virus suspension qualified in control tests shall be purified by means of chromatography or other appropriate methods. A suitable amount of human albumin can be added as a stabilizer and thimerosal added as a preservative to make the bulk.

2.3.9 Control tests on bulk

See Section 3.2.

2.4 Final bulk

2.4.1 Formulation

Formulation shall be carried out according to the protein and antigen contents. The total protein content of each single human dose shall be not more than 120 µg. It is defined as the final bulk.

2.4.2 Control tests on final bulk

See Section 3.3.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

0.5 ml of reconstituted vaccine per container.
0.5 ml per single human dose. The potency of the vaccine shall be not less than 2.5 IU.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on single virus harvest

3.1.1 Virus titration

See Section 2.2.3.2. The titer shall be not less than 6.0 lg LD₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control tests on bulk

3.2.1 Determination of antigen content

A suitable method shall be adopted for determination of antigen content.

3.2.2 Validation test for effective inactivation

The test sample shall be inoculated i. c. into twenty mice each weighing 11-13 g, 0.03 ml for each. Observe the animals for 14 days. All of them shall survive the observation period. Those that die

within 3 days after injection shall be excluded from the final evaluation.

3.2.3 Protein content

Sample shall be collected from purified preparation prior to adding human blood albumin. The content shall be not more than 120 µg/dose (Appendix VI B, method 2).

3.2.4 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.2.5 Determination of residual DNA content of Vero cells

The residual DNA content of Vero cells shall be not more than 100 pg/dose (Appendix IX B).

3.2.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.4 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.4.1 Identity test

See Section 3.4.4. The test shall be considered unsatisfactory if the potency test fails.

3.4.2 Inspection on final containers

The vaccine looks like a white crisp cake. The reconstituted vaccine is a clear liquid, free of foreign matters.

3.4.3 Chemical tests

3.4.3.1 pH

The pH shall be 7.2-8.0 (Appendix V A).

3.4.3.2 Moisture content

The content of residual moisture shall be not more than 3.0% (Appendix VII D).

3.4.3.3 Content of thimerosal

The content of thimerosal shall be not more than 0.10 mg/ml (Appendix VII B).

3.4.4 Potency test

The potency of the vaccine shall be not less than 2.5 IU/dose (Appendix XI A).

3.4.5 Thermostability test

Before release each lot of vaccine shall be subject to thermostability test. The test sample is exposed at 37°C for 14 days for the determination of potency (See Section 3.4.4). The test vaccine which passed the thermostability test is considered as satisfactory in potency test.

3.4.6 Sterility test

It complies with the test for sterility (Appendix

Ⅺ A).

3.4.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix Ⅺ F).

3.4.8 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU/dose (Appendix Ⅺ E, the limit test of gel-clot method).

4 Storage, shipping and validity period

Store and ship at or below 8°C, protected from light. The validity period is 18 months starting from the date when potency test proved qualified.

5 Package inserts

Directions for Use of Rabies Vaccine (Vero Cell) for Human Use, Freeze-dried

[Drug Name]

Adopted name: Rabies Vaccine (Vero Cell) for Human Use, Freeze-dried

[Constituents and characters]

The vaccine is a freeze-dried preparation of rabies fixed virus grown in Vero cells. After cultivation and harvest, the virus suspension is inactivated, concentrated and purified, and lyophilized after the addition of a suitable stabilizer. It looks like a white crisp cake. After reconstitution it turns into a clear liquid containing thimerosal as a preservative.

[Eligibles]

If a person is bitten or scratched by a rabid dog or other rabid animals, regardless of age or sex, the wounds shall be cleaned immediately (flush the wounds repeatedly with clean water or soap water, followed by applying iodine tincture or ethanol for several times), and the exposed person shall be inoculated with the vaccine according to the post-exposure schedule as soon as possible. The persons at risk of contacting rabies virus (such as veterinarians, animal breeders, forestry workers, workers in slaughterhouse and staffs in rabies laboratory) shall be immunized following the pre-exposure treatment schedule.

[Function and use]

The preparation can induce immunity against rabies virus in recipients following immunization. It is used to prevent rabies in human.

[Specifications]

0.5 ml of reconstituted vaccine per container. 0.5 ml per single human dose. The potency of the vaccine shall be not less than 2.5 IU.

[Administration and dosage]

(1) Reconstitute the vaccine with stated amount of sterile water for injection, shake the container well, the content is reconstituted completely before use.

(2) The deltoid muscle of the upper arm is the recommended site for i.m. administration. For young children, inoculate the vaccine in the muscle at

anterolateral aspect of the thigh.

(3) Post-exposure schedule for immunization: Normally one dose of the vaccine shall be administered to the exposed person on days 0 (the first day or the intraday), 3 (the fourth day, analogically henceforth), 7, 14, and 28, consecutively, five doses in total. Children shall be treated in the same way. It is recommended to double the first dose of vaccine in case of one of the following situations:

①The exposed person was injected with immunoglobulin or antiserum one month before the day of receiving rabies vaccine.

②Those with congenital or acquired immunodeficiency.

③Those receiving immunosuppressant (including antimalaria drug).

④The elderly or patients with chronic diseases.

⑤Administration of rabies vaccine becomes available to the exposed persons 48 hours or longer after exposure.

Post-exposure treatment shall be dependent on the following classification of wound severity:

Category I: those petting animal, licked by animal on intact skins without any breaks—neither wound treatment nor administration of vaccine is necessary.

Category II: those bitten or scratched by animal on skins but without bleeding; or licked on skins with breaks—vaccine shall be administered following the post-exposure immunization schedule.

Category III: those with single or multiple biting wounds on skins with bleeding or scratched with bleeding; mucous membrane was contaminated by saliva of suspected or confirmed rabid animal—the exposed person shall be treated immediately with rabies vaccine, rabies antiserum (40 IU/kg, horse origin) or immunoglobulin (20 IU/kg, human origin). If anatomically feasible, infiltration injection of the remaining serum (horse or human origin) shall be performed as much as possible around the wound (s); the rest, if any, shall be injected intramuscularly.

(4) Preexposure immunization schedule: A total of three shots given on days 0, 7 and 28.

(5) Recommendation of boosters for those immunized with rabies vaccine previously:

①Complete post-exposure immunization course was conducted in the recent one year; if bitten by a suspected rabid animal, one dose given on days 0 and 3 separately.

②Complete post-exposure immunization course was conducted in the previous year; if bitten by a suspected rabid animal, carry out a complete immunization course again.

③Complete post-exposure immunization course was conducted in the last 3 years, and followed by boosters; if bitten by a suspected rabid animal, one dose of vaccine shall be given on days 0 and 3 separately.

④Complete post-exposure immunization course



and booster (s) was conducted more than 3 years ago; if bitten by a suspected rabid animal, a complete post-exposure immunization course shall be executed.

[Adverse reactions]

After inoculation, mild local or systemic reactions may occur, which could be relieved spontaneously. Occasionally rashes may appear. In case of some serious adverse reactions, such as immediate anaphylactic reactions, angioneurotic edema or urticaria, symptomatic treatment is recommended.

[Contraindications]

- (1) Because rabies is a fatal disease, there are no contraindications for post-exposure immunization.
- (2) For preexposure immunization, it is not recommended to immunize eligible individuals with fever, acute disease, serious chronic disease, nervous system disease, and with a history of allergic reaction to antibiotics and/or biological products. It is recommended to postpone the administration of the vaccine for women in pregnancy or in lactation, if feasible.

[Precautions]

- (1) Do not use the vaccine if the reconstituted vaccine contains any foreign substance, or any leakage of container or illegible label is found.
- (2) Alcoholic drinks, strong tea, pungent food and strenuous exercise shall be avoided after injection of the vaccine.
- (3) Do not inject the vaccine in the gluteal region.

[Storage]

Store and ship at or below 8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name;
Address;
Zip code;
Tel;
Fax;
Web site;

Rabies Vaccine (Hamster Kidney Cell) for Human Use

The rabies vaccine is a preparation of rabies fixed virus grown in primary hamster kidney cell cultures. After cultivation and harvest, the virus suspension is inactivated, concentrated and purified before adding a suitable stabilizer to make the

vaccine. Aluminum hydroxide may be added as an adjuvant. It is used to prevent rabies in human.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Primary hamster kidney cell cultures shall be used for the vaccine production.

2.1.1 Management and quality control on cell substrates

The Requirements for Preparation and Control of Animals Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

The kidneys of hamster of 12-14 days old are extracted aseptically. The tissue fragments are digested and dispersed with trypsin solution. The dispersed cells are distributed into cell culture bottles. The cells prepared in one container are defined as a cell digested batch.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Strain aG or other rabies fixed virus strains adapted in hamster kidney cells can be used for the vaccine production.

2.2.2 Establishment of virus seed lot

The Requirements for Bacterial and Viral Strains Used for Manufacture and Quality Control of Biologics shall apply.

The primary seed lot is 2aG1. The master seed lot shall not exceed 4aG. The working seed lot is prepared by alternative subcultures of the virus on primary hamster kidney cells and in guinea pig brains. The virus passage shall not exceed the 6th passage on primary hamster kidney cells, i.e. not exceed 10aG, and not exceed the 5th passage in brains of guinea pig, i.e. 10aG5.

2.2.3 Control tests on virus seed lots

For master seed lots, comprehensive control tests described below shall be carried out; for working seed lots, tests described in Sections 2.2.3.1-2.2.3.5 shall be carried out at least.

2.2.3.1 Identity test

Neutralization test by i.c. route in mice shall be carried out to identify the specificity of the virus seed. The neutralization index shall be not less than 500.

2.2.3.2 Virus titration

Dilute the sample of seed virus 10-fold serially. Inoculate i.c. each of at least six mice each weighing 11-13 g with the virus suspension of each dilution. The titer shall be not less than 8.0 lg LD₅₀/ml.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix XIII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XIII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix XIII C).

2.2.3.6 Tests for immunogenicity

Prepare the original vaccine with the seed virus of master seed lot to immunize i. p. a group of mice each weighing 12-14 g. Each animal receives 0.5 ml twice at an interval of one week. Two weeks after the first inoculation, challenge intracerebrally the immunized mice individually with 0.03 ml of CVS virus diluted 10-fold serially and ten mice are required for each dilution. The CVS virus shall be titrated in parallel in mice with the same weight as a control, ten mice are required for each dilution. The protective index of the virus seed shall be not less than 100.

2.2.4 Storage of virus seeds

Freeze-dried virus seed lots shall be stored at or below -60°C . The liquid working seed lots shall be stored at or below -60°C for not more than 2 years.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Lacto-albumin hydrolysate, MEM, medium 199 or other suitable media containing a quantity of inactivated calf serum is used as the culture medium. The quality of calf serum shall comply with the requirements in Appendix XIII D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix XIII C).

2.3.4 Virus inoculation and cultivation

Inoculate rabies seed virus onto confluent monolayer of cell cultures. Incubate the cultures at a suitable temperature for a certain period of time. Discard the culture medium and flush the cell sheets with PBS to remove calf serum. A quantity of maintenance medium is added to continue the cultivation process at $33-35^{\circ}\text{C}$.

2.3.5 Virus harvest

After cultivation for a certain period of time, harvest the virus suspension, i. e. the single viral harvest. Multiple harvests can be achieved depending on the situation of cell growth.

2.3.6 Control tests on single harvest

See Section 3.1.

2.3.7 Virus inactivation

Formalin shall be added to the pool of viral

harvests in a proportion of 1 : 4000. Inactivation shall be carried out at a suitable temperature for a period of time.

2.3.8 Concentration and purification

2.3.8.1 Pooling and concentration by

A number of single harvests derived from the same lot of cell cultures can be pooled into one batch following strictly aseptic procedures. The yield shall be properly concentrated by means of ultrafiltration.

2.3.8.2 Purification

The concentrated virus suspension qualified in control tests shall be purified by means of chromatography or other appropriate methods. A suitable amount of human albumin shall be added as a stabilizer and thimerosal added as a preservative to make the bulk.

2.3.9 Control tests on bulk

See Section 3.2.

2.4 Final bulk

2.4.1 Formulation

Formulation shall be carried out according to its protein and antigen contents. The protein content of each single human dose shall be less than $120\text{ }\mu\text{g}$. Aluminum hydroxide may be added as an adjuvant to prepare the final bulk; its final concentration shall be not more than 0.7 mg/ml .

2.4.2 Control tests on final bulk

See Section 3.3.

2.5 Final product

2.5.1 Batch defining

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1.0 ml per container, 1.0 ml per single human dose. The potency of the vaccine shall be not less than 2.5 IU.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on single virus harvests

3.1.1 Virus titration

See Section 2.2.3.2. The titer shall be not less than $5.5\text{ lg LD}_{50}/\text{ml}$.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XIII B).



3.2 Control tests on bulk

3.2.1 Determination of antigen content

A suitable method shall be adopted for determination of antigen content.

3.2.2 Validation test for effective inactivation

The test sample shall be inoculated i. c. into twenty mice each weighing 11-13 g, 0.03 ml for each. Observe the animals for 14 days. All of them shall survive the observation period. Those that die within 3 days after injection shall be excluded from the final evaluation.

3.2.3 Protein content

Sample shall be collected from purified preparation prior to adding human blood albumin. The content shall be not more than 120 µg/dose (Appendix VI B, method 2).

3.2.4 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.2.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.4 Control tests on final product

3.4.1 Identity test

See Section 3.4.4. The test shall be considered unsatisfactory if the potency test fails.

3.4.2 Inspection on final containers

The vaccine is a turbid, milky-white liquid. After storage for a long time, a precipitate may form which can be dispersed on shaking. It should be free of foreign matters. The vaccine without adjuvant shall be a clear, colourless liquid.

3.4.3 Chemical tests

3.4.3.1 pH

The pH shall be 7.2-8.0 (Appendix V A).

3.4.3.2 Content of thimerosal

The thimerosal content shall be not more than 0.10 mg/ml (Appendix VII B).

3.4.3.3 Content of aluminum hydroxide

The vaccine with adjuvant should be subject to this test. The content of aluminum hydroxide shall be not more than 0.70 mg/ml (Appendix VII F).

3.4.3.4 Content of free formaldehyde

The content of free formaldehyde shall be not more than 0.10 mg/ml (Appendix VI L).

3.4.4 Potency test

The potency of the vaccine shall be not less than 2.5 IU/dose (Appendix XI A).

3.4.5 Thermostability test

Before release each lot of vaccine shall be subject to

thermostability test. The test sample is exposed at 37°C for 14 days for the determination of potency (See Section 3.4.4). The test vaccine which passed the thermostability test is considered satisfactory in potency test.

3.4.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.4.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.4.8 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU/dose (Appendix XII E, the limit test of gel-clot method).

4 Storage, shipping and validity period

Store and ship at 2-8°C and protected from light. The validity period is 12 months starting from the date when potency test proved qualified.

5 Package inserts

Directions for Use of Rabies Vaccine (Hamster Kidney Cell) for Human Use

[Drug name]

Adopted name: Rabies Vaccine (Hamster Kidney Cell) for Human Use

[Constituents and characters]

The vaccine is a preparation of rabies fixed virus grown in primary hamster kidney cell cultures. After cultivation and harvest, the virus suspension is inactivated, concentrated and purified before adding a suitable stabilizer. Aluminum hydroxide may be added as an adjuvant. The vaccine with adjuvant is a turbid, milky-white liquid, and the vaccine without adjuvant is a colourless clear liquid. Both of them contain thimerosal as a preservative.

[Eligibles]

If a person is bitten or scratched by a rabid dog or other rabid animals, regardless of age or sex, the wounds shall be cleaned immediately (flush the wounds repeatedly with clean water or soap water, followed by applying iodine tincture or ethanol for several times), and the exposed person shall be inoculated with the vaccine according to the post-exposure schedule as soon as possible. The persons at risk of contacting rabies virus (such as veterinarians, animal breeders, forestry workers, workers in slaughterhouse and staffs in rabies laboratory) shall be immunized following the pre-exposure treatment schedule.

[Function and use]

The preparation can induce immunity against rabies virus in recipients following immunization. It is used to prevent rabies in human.

[Specifications]

1.0 ml per container. 1.0 ml per single human dose. The potency of the vaccine shall be not less

than 2.5 IU.

[Administration and dosage]

(1) The deltoid muscle of the upper arm is the recommended site for i.m. administration. For young children, inoculate the vaccine in the muscle at anterolateral aspect of the thigh.

(2) Post-exposure schedule for immunization: Normally one dose of the vaccine shall be administered to the exposed person on days 0 (the first day or the intraday), 3 (the fourth day, analogically henceforth), 7, 14, and 28, consecutively, five doses in total. Children shall be treated in the same way. It is recommended to double the first dose of vaccine in case of one of the following situations:

①The exposed person was injected with immunoglobulin or antiserum one month before the day of receiving rabies vaccine.

②Those with congenital or acquired immunodeficiency.

③Those receiving immunosuppressant (including antimalaria drug).

④The elderly or patients with chronic diseases.

⑤Administration of rabies vaccine becomes available to the exposed persons 48 hours or longer after exposure.

Post-exposure treatment shall be dependent on the following classification of wound severity:

Category I: those petting animal, licked by animal on intact skins without any breaks—neither wound treatment nor administration of vaccine is necessary.

Category II: those bitten or scratched by animal on skins but without bleeding; or licked on skins with breaks—vaccine shall be administered following the post-exposure immunization schedule.

Category III: those with single or multiple biting wounds on skins with bleeding or scratched with bleeding; mucous membrane was contaminated by saliva of suspected or confirmed rabid animal—the exposed person shall be treated immediately with rabies vaccine, rabies antiserum (40 IU/kg, horse origin) or immunoglobulin (20 IU/kg, human origin). If anatomically feasible, infiltration injection of the remaining serum (horse or human origin) shall be performed as much as possible around the wound (s); the rest, if any, shall be injected intramuscularly.

(3) Preexposure immunization schedule: A total of three shots given on days 0, 7 and 28.

(4) Recommendation of boosters for those immunized with rabies vaccine previously:

①Complete postexposure immunization course was conducted in the recent one year; if bitten by a suspected rabid animal, one dose given on days 0 and 3 separately.

②Complete post-exposure immunization course was conducted in the previous year; if bitten by a suspected rabid animal, carry out a complete immunization course again.

③Complete post-exposure immunization course

was conducted in the last 3 years, and followed by boosters: if bitten by a suspected rabid animal, one dose of vaccine shall be given on days 0 and 3 separately.

④Complete post-exposure immunization course and booster (s) was conducted more than 3 years ago; if bitten by a suspected rabid animal, a complete post-exposure immunization course shall be executed.

[Adverse reactions]

After inoculation, mild local or systemic reactions may occur, which could be relieved spontaneously. Occasionally rashes may appear. In case of some serious adverse reactions, such as immediate anaphylactic reactions, angioneurotic edema or urticaria, symptomatic treatment is recommended.

[Contraindications]

(1) Because rabies is a fatal disease, there is no contraindications for post-exposure immunization.

(2) For preexposure immunization, it is not recommended to immunize eligible individuals with fever, acute disease, serious chronic disease, nervous system disease, and with a history of allergic reaction to antibiotics and/or biological products. It is recommended to postpone the administration of the vaccine for women in pregnancy or in lactation, if feasible.

[Precautions]

(1) Do not use the vaccine if the vaccine contains any foreign substance, or any leakage of container or illegible label is found.

(2) Alcoholic drinks, strong tea, pungent food and strenuous exercise shall be avoided after injection of the vaccine.

(3) Do not inject the vaccine in the gluteal region.

(4) Freezing is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

12 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Measles Vaccine, Live

Measles vaccine is a preparation of live attenuated measles virus grown in chick primary embryo cell



cultures. After cultivation and harvest, the virus suspension, after adding a suitable stabilizer, is lyophilized to make the vaccine. It is used to prevent measles.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for the vaccine production

Primary chick embryo cells are used for the preparation of virus seeds and vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

The chick embryo of 9-11 days old from SPF stocks shall be selected and trypsinized to prepare dispersed cells which are then cultivated with an appropriate medium.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Strain Hu-191, strain Chang-47 or other approved attenuated measles virus strains can be used as the viral seed for the vaccine production.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

The vaccine production shall be based on a seed lot system. The final product derived from strain Hu-191 shall not exceed the 33rd passage and the final product derived from strain Chang 47 shall not exceed the 41st passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Dilute the sample of seed virus to make virus suspension at a concentration of 500-2000 CCID₅₀/ml. Mix the virus suspension with an equal volume of measles antiserum. Keep the mixture in 37°C water bath for 60 minutes. Inoculate the incubated mixture onto FL or Vero cells. Evaluate results after incubation for 7-8 days at a suitable temperature. Measles virus shall be completely neutralized (no CPE observed); meanwhile, both the results of the serum control and the cell control shall be negative. The titer of reference virus preparation shall be not less than 500 CCID₅₀/ml.

2.2.3.2 Virus titration

Dilute the sample of virus seed 10-fold serially.

Inoculate the virus suspension at different dilutions onto FL or Vero cell cultures. The results are evaluated after incubation at a suitable temperature for 7-8 days. The titer shall be not less than 4.5 lg CCID₅₀/ml. Titration of the virus reference preparation shall be carried out in parallel.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix XII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix XII C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the master seed virus to immunize at least thirty healthy and susceptible children. Blood samples are taken for determination of measles antibody before and 4-6 weeks after the inoculation, respectively. The antibody conversion rate shall be not less than 95% (HI titer <1 : 2 or ELISA <1 : 200 as negative; HI titer ≥1 : 2 or ELISA ≥1 : 200 as positive).

2.2.3.7 Test for neurovirulence in monkeys

The master seed lot or working seed lot shall be demonstrated to be free from neurovirulence. At least ten monkeys, serologically negative for measles immediately prior to the neurovirulence test, shall be employed for each time. The test sample shall be given to each monkey by inoculation of 0.5 ml (representing not less than the virus content of one single human dose of vaccine) into the thalamic region of each hemisphere. The monkeys shall be observed for 17-21 days. No symptoms and signs of paralysis or any evidences of neurological involvement shall be found. Monkeys, if not more than two, that died within 48 hours after injection may be replaced. The test is invalid and shall be repeated if more than 20% of the monkeys die even due to nonspecific causes. At the end of the observation period each monkey is bled and the individual sera shall be tested for measles antibody. The seroconversion rate shall be not less than 80%. Each monkey is subjected to autopsy. Histopathological examinations of appropriate areas of the brain and spinal cord shall be made for any evidence of central nervous system involvement. The results shall be negative. Meanwhile, at least four measles-susceptible monkeys shall be reserved as controls. Blood samples shall be taken from the control monkeys at the time of inoculating the test monkeys, and on the 10th day after the test animals are killed. All the serum samples from the control monkeys shall remain free from measles antibody.

2.2.4 Storage of virus seed lots

The freeze-dried virus seed lots shall be stored at

or below -20°C , the liquid ones shall be stored at or below -60°C .

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Earle solution containing lacto-albumin hydrolysate and a quantity of inactivated calf serum can be used as the culture medium. Other suitable culture media can also be used. The quality of calf serum shall comply with the related requirements in Appendix VIII D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix VIII C).

2.3.4 Virus inoculation and cultivation

The mixture of the virus and the dispersed cells at an optimal MOI shall be inoculated in the culture vessels which are then incubated at a suitable temperature. Discard the culture medium when the CPE develops to a certain extent. Flush the cell sheets with a rinsing solution of which the volume in each vessel shall be not less than that of the original culture medium. Maintenance medium is added after rinsing to continue the cultivation.

2.3.5 Virus harvest

Harvest the virus suspension when the CPE reaches to a certain extent. After harvest, the culture may be further incubated for multiple harvests by replacement with fresh maintenance medium.

2.3.6 Pooling

A number of single harvests derived from the cultures made of the same lot of dispersed cells can be pooled into one batch of bulk.

2.3.7 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

The bulk can be properly diluted according to the virus titer. The final bulk shall be prepared by adding a quantity of stabilizer. A number of bulks can be pooled into one batch of final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. During the filling process, the final bulk shall be kept cool in ice bath.

2.5.3 Specifications

0.5 ml, 1.0 ml or 2.0 ml of reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 3.0 lg CCID₅₀ of live measles virus.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Virus titration

See Section 2.2.3.2. The titer shall be not less than 4.5 lg CCID₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix VIII B).

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

See Section 2.2.3.1

3.3.2 Inspection on final containers

The product looks like a milky-white crisp cake. After reconstitution, it shall turn into a clear liquid, orange-red or pale pink in colour, free of foreign matters.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Virus titration

See Section 2.2.3.2. A mixture of three to five containers of vaccine shall be made for virus titration. The titer shall be not less than 3.3 lg CCID₅₀/ml.

3.3.5 Thermostability test

Before release the final product shall be subject to thermostability test which shall be performed at the same time with virus titration in parallel. The vaccine samples that have been exposed at 37°C for 7 days shall be titrated following Section 2.2.3.2. The virus titer shall be not less than 3.3 lg CCID₅₀/ml. The loss of virus titer of the heat exposed vaccine shall be not more than 1.0 lg.

3.3.6 Content of residual bovine serum albumin

The residual bovine serum albumin content shall be not more than 50 ng/dose determined by ELISA.

3.3.7 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

4 Storage, shipping and validity period

Store and ship at or below 8°C, protected from light. The validity period of measles vaccine is 18 months starting from the date when titration test proved qualified.

5 Package inserts

Directions for Use of Measles Vaccine, Live

[Drug name]

Adopted name: Measles Vaccine, Live

[Constituents and characters]

Measles vaccine is a preparation of live attenuated measles virus grown in chick primary embryo cell culture. After cultivation and harvest, the virus suspension, after adding a suitable stabilizer, is lyophilized to make the vaccine. The vaccine looks like a milky-white crisp cake. After reconstitution, it turns into a clear liquid, orange-red or pale pink in colour.

[Eligibles]

Children susceptible to measles at or above 8 months of age.

[Function and use]

The product can induce immunity against measles virus in recipients following immunization. It is used to prevent measles.

[Specifications]

0.5 ml, 1.0 ml or 2.0 ml of reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 3.0 lg CCID₅₀ of live measles virus.

[Administration and dosage]

(1) Reconstitute the vaccine with the stated amount of sterile water for injection. Shake the container till the content is reconstituted completely before use.

(2) Inject s.c. 0.5 ml of the vaccine at deltoid insertion area of the lateral upper arm.

[Adverse reactions]

Normally there are no local reactions at the inoculation site. A small number of recipients might have transient fever or scattered skin rashes during the period between 6 and 10 days after injection. The reactions would not last longer than 2 days and could be relieved spontaneously. No particular treatment is necessary. Symptomatic treatment could be adopted in case of need.

[Contraindications]

(1) Subjects with serious diseases, acute or chronic infections or fever.

(2) Those with a history of allergic reaction to eggs.

(3) Women in pregnancy.

[Precautions]

(1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.

(2) Do not use the vaccine if any leakage of container or illegible label is found, or the content is not transparent after reconstitution.

(3) The vaccine should be kept at 2-8°C and used up within one hour after reconstitution; the remaining vaccine, if any, shall be discarded.

(4) The immunization of measles vaccine should be deferred for at least one month following administration of immunoglobulin.

[Storage and shipping]

Store and ship at or below 8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Rubella Vaccine (Human Diploid Cell), Live

This rubella vaccine is a freeze-dried preparation of live attenuated rubella virus grown in human diploid cells. After cultivation and harvest, the virus suspension is lyophilized to make the vaccine after the addition of a suitable stabilizer. It is used to prevent rubella.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for production

The human diploid cell strains (2BS, MRC-5 or any approved cell strains) shall be used for production of the vaccine.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

The cell cultures which are derived from the cells, through resurrection and propagation, of one or several ampoules from the same lot of working cell bank shall only be used for the production of one lot of vaccine.

For 2BS cell strain, the maximum number of population doublings for the master cell bank shall be the 23rd, for working cell bank shall be the 27th, for the production of the vaccine shall be the 44th.

For MRC-5 cell strain, the maximum number of population doublings for the master cell bank shall be the 23rd; for the working cell bank shall be the 27th, for the production of the vaccine shall be the 33rd.

2.1.2 Cell substrate preparation

Cells in one or more ampoules taken from working cell bank each time are resurrected and expanded to produce a collection of cell cultures enough for virus inoculation in production. The cell cultures are regarded as a cell lot.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Strain BRD II or other approved attenuated rubella virus strains adapted in diploid cells can be used as the seed for the vaccine production.

2.2.2 Establishment of viral seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

The vaccine production shall be based on a seed lot system. The final product derived from the strain BRD II shall not exceed the 32nd passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Dilute the sample of seed virus to make a concentration of 100-500 CCID₅₀/ml. Mix the virus suspension with an equal volume of appropriately diluted rubella antiserum. Keep the mixture in 37°C water bath for 60 minutes. Inoculate the incubated mixture onto RK-13 cells. Evaluate the results after incubation at 32°C for 7-10 days. Rubella virus shall be completely neutralized (no CPE observed); meanwhile, both the results of serum control and cell control shall be negative. The titer of reference virus preparation shall be not less than 100 CCID₅₀/ml.

2.2.3.2 Virus titration

Dilute the sample of virus seed 10-fold serially. Inoculate the virus suspension at different dilutions onto RK-13 cell cultures. The results are evaluated after incubation at 32°C for 7-10 days. The titer shall be not less than 4.8 lg CCID₅₀/ml. Titration of the virus reference preparation shall be

carried out in parallel.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix III A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix III C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the virus seed from the master seed lot to immunize at least thirty healthy children susceptible to rubella. Blood samples are taken respectively before and 4-6 weeks after inoculation to determine rubella antibody. The antibody conversion rate shall be not less than 95% (HI titer <1 : 8 as negative; HI titer ≥1 : 8 as positive).

2.2.3.7 Test for neurovirulence in monkeys

The master seed lot or working seed lot shall be demonstrated to be free from neurovirulence. At least ten monkeys, serologically negative for rubella immediately prior to the neurovirulence test, shall be employed for the test. The test sample shall be given to each monkey by inoculation of 0.5 ml (equivalent to not less than the virus content of one single human dose of the vaccine) into the thalamic region of each hemisphere. The monkeys shall be observed for 17-21 days. No symptoms and signs of paralysis or any evidences of neurological involvement shall be found. The vacancies of monkeys not more than two that die within 48 hours after injection, can be replaced. The test is invalid and shall be repeated if more than 20% of the monkeys die even due to nonspecific causes. At the end of the observation period each monkey is bled and the individual sera shall be tested for rubella antibody. The seroconversion rate shall be not less than 80%. Each monkey is subjected to autopsy. Histopathological examinations of appropriate areas of the brain and spinal cord shall be made for any evidence of central nervous system involvement. The results shall be negative. Meanwhile, at least two rubella-susceptible monkeys shall be reserved as controls. Blood samples shall be taken from the control monkeys at the time of inoculating the test monkeys, and on the 10th day after the test animals are killed. All the serum samples from the control monkeys shall remain free from rubella antibody.

2.2.4 Storage of virus seed lots

The freeze-dried virus seed lots shall be stored at or below -20°C, the liquid working virus seed lots shall be stored at or below -60°C.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2. 1. 2.

2. 3. 2 Culture medium

MEM containing a quantity of inactivated calf serum can be used. Other suitable culture media can also be used. The quality of calf serum shall comply with the requirements in Appendix VIII D.

2. 3. 3 Tests for adventitious viruses in control cells
It complies with the tests for adventitious viruses (Appendix VIII C).

2. 3. 4 Virus inoculation and cultivation
Inoculate the virus onto the cell cultures at an optimal MOI. Incubate the cultures at 30-32°C. Discard the culture medium when the CPE develops to a certain extent. Flush the surface of cell sheets with a rinsing solution of which the volume in each vessel shall be not less than that of the original culture medium. Maintenance medium is added after rinsing to continue the cultivation.

2. 3. 5 Virus harvest
Harvest the virus suspension when the CPE reaches to a certain extent. After harvest, the culture may be further incubated for multiple harvests by replacement with fresh maintenance medium.

2. 3. 6 Pooling
A number of single harvests derived from the same batch of cell cultures can be pooled into one batch.

2. 3. 7 Control tests on bulk
See Section 3. 1.

2. 4 Final bulk

2. 4. 1 Formulation
The bulk can be properly diluted according to the virus titer. The final bulk is prepared by adding a quantity of stabilizer. A number of bulks can be pooled into one batch of final bulk.

2. 4. 2 Control tests on final bulk
See Section 3. 2.

2. 5 Final product

2. 5. 1 Defining batches
The Requirements for Defining Batches of Biologics shall apply.

2. 5. 2 Filling and lyophilization
The Requirements for Filling and lyophilization of Biologics shall apply. During the filling process, the final bulk shall be kept cool in ice bath.

2. 5. 3 Specifications
0. 5 ml or 1. 0 ml of the reconstituted vaccine per container. 0. 5 ml per single human dose containing not less than 3. 2 lg CCID₅₀ of live rubella virus.

2. 5. 4 Packaging
The Requirements for Packaging of Biologics shall apply.

3 Control tests

3. 1 Control tests on bulk

3. 1. 1 Virus titration

See Section 2. 2. 3. 2. The titer shall be not less than 4. 8 lg CCID₅₀/ml.

3. 1. 2 Sterility test

It complies with the test for sterility (Appendix VIII A).

3. 1. 3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix VIII B).

3. 2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix VIII A).

3. 3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3. 3. 1 Identity test

See Section 2. 2. 3. 1.

3. 3. 2 Inspection on final containers

The product looks like a milky-white, crisp cake. After reconstitution, it shall turn into a clear, orange-red liquid, free of foreign matters.

3. 3. 3 Moisture content

The residual moisture content shall be not more than 3. 0% (Appendix VII D).

3. 3. 4 Virus titration

See Section 2. 2. 3. 2. A mixture of three to five containers of vaccine shall be made for virus titration. The titer shall be not less than 3. 5 lg CCID₅₀/ml.

3. 3. 5 Thermostability test

Before release the final product shall be subject to thermostability test which shall be performed at the same time with virus titration in parallel. The vaccine samples that have been exposed at 37°C for 7 days shall be titrated following Section 2. 2. 3. 2. The virus titer shall be not less than 3. 5 lg CCID₅₀/ml. The loss of virus titer of the heat exposed vaccine shall be not more than 1. 0 lg.

3. 3. 6 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3. 3. 7 Sterility test

It complies with the test for sterility (Appendix VIII A).

3. 3. 8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

4 Storage, shipping and validity period

Store and ship at or below 8°C, protected from light. The validity period of rubella vaccine is 18 months starting from the date when virus titration test proved qualified.

5 Package inserts

Directions for Use of Rubella Vaccine (Human Diploid Cell), Live

[Drug name]

Adopted name: Rubella Vaccine (Human Diploid Cell), Live

[Constituents and characters]

The vaccine is made from an attenuated rubella virus strain grown in human diploid cell cultures. After cultivation, the virus suspension is harvested and an appropriate stabilizer is added for lyophilization. The freeze-dried vaccine looks like a milky-white, crisp cake. After reconstitution, it shall turn into a clear, orange-red liquid.

[Eligibles]

Children susceptible to rubella at or above 8 months of age.

[Function and use]

The product can induce immunity against rubella virus in recipients following immunization. It is used to prevent rubella.

[Specifications]

0.5 ml or 1.0 ml of the reconstituted vaccine per container: 0.5 ml per single human dose containing not less than 3.2 lg CCID₅₀ of live rubella virus.

[Administration and dosage]

(1) Reconstitute the vaccine with the stated amount of sterile water for injection. Shake the container till the content is reconstituted completely before use.

(2) Inject s.c. 0.5 ml of the vaccine at deltoid insertion area of the lateral upper arm.

[Adverse reactions]

Normally there are no local reactions at the inoculation site. A small number of recipients might have transient fever or mild skin rashes during the period between 6 and 11 days after injection. The reactions would not last longer than 2 days and could be relieved spontaneously. Some adult individuals may have mild arthralgia 2-4 weeks after injection. No particular treatment is necessary. Symptomatic treatment could be adopted in case of need.

[Contraindications]

- (1) Subjects with serious diseases, or fever;
- (2) Those with a history of allergic reaction;
- (3) Women in pregnancy.

[Precautions]

- (1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.
- (2) Do not use the vaccine if any leakage of container or illegible label is found, or the content of the vaccine can not be completely reconstituted.
- (3) The vaccine should be kept at 2-8°C and used up within one hour after reconstitution; the

remaining vaccine, if any, shall be discarded.

(4) Women of childbearing age shall take some contraceptive measures for at least 3 months after immunization.

(5) The immunization of rubella vaccine should be deferred for at least one month following administration of immunoglobulin.

(6) Do not receive other live vaccines one month before or after administering this vaccine. However, the vaccine can be given with live measles and mumps vaccines concomitantly.

[Storage and shipping]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Rubella Vaccine (Rabbit Kidney Cell), Live

This rubella Vaccine is a freeze-dried preparation of live attenuated rubella virus grown in primary rabbit kidney cells. After cultivation and harvest, the virus suspension is lyophilized to make the vaccine after the addition of a suitable stabilizer. It is used to prevent rubella.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for production

Primary rabbit kidney cells are used for production of the vaccine.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

Rabbits of 25-30 days old which shall satisfy the standards for clean animals shall be used. Kidneys are extracted aseptically and digested with trypsin solution. The trypsinized primary rabbit kidney

cells shall be cultivated at 37°C or at a suitable temperature.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Matsuba strain is used as the seed for the vaccine production.

2.2.2 Establishment of viral seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

The vaccine production shall be based on a seed lot system. The final product shall not exceed the 19th passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Properly diluted rubella serum and a quantity of properly diluted rubella virus are mixed in equal volume and incubated in 37°C water bath for 60 minutes. Inoculate the incubated mixture onto RK-13 cell cultures. Evaluate the results after incubation at 32°C for 14 days. Rubella virus shall be completely neutralized; no CPE shall be observed. The serum control and the cell control are tested in parallel; both of result shall be negative. The virus control shall manifest typical rubella CPE.

2.2.3.2 Virus titration

Dilute the sample of virus seed 10-fold serially. Inoculate the virus suspensions at different dilutions onto RK-13 cell cultures. The results are evaluated after incubation at 32°C for 14 days. The titer shall be not less than 4.8 lg CCID₅₀/ml. Titration of the virus reference preparation shall be carried out in parallel.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix III A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix III C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the virus seed from the master seed lots to immunize at least thirty healthy children susceptible to rubella. Blood samples are taken respectively before and 4-6 weeks after inoculation to determine rubella antibody. The antibody conversion rate shall be not less than 95% (HI titer < 1 : 8 as negative; HI titer ≥ 1 : 8 as positive).

2.2.3.7 Neurovirulence test in monkeys

The master seed lot or working seed lot shall be demonstrated to be free from neurovirulence. At least ten monkeys, serologically negative (HI titer < 1 : 8) for rubella immediately prior to the neurovirulence test, shall be employed for the test. The test sample shall be given to each monkey by inoculation of 0.5 ml (equivalent to not less than the virus content of one single human dose of the vaccine) into the thalamic region of each hemisphere. The monkeys shall be observed for 17-21 days. The vacancies of monkeys that die within 48 hours after injection can be replaced. The test is invalid and shall be repeated if more than 20% of the monkeys die even due to nonspecific causes. At the end of the observation period each monkey is bled and the individual sera shall be tested for rubella antibody. The seroconversion rate shall be not less than 80%. Each monkey is subjected to autopsy. Histopathological examinations of appropriate areas of the brain and spinal cord shall be made for any evidence of central nervous system involvement. The results shall be negative. Meanwhile, at least two rubella-susceptible monkeys shall be reserved as controls. Blood samples shall be taken from the control monkeys at the time of inoculating the test monkeys, and on the 10th day after the test animals are killed. All the serum samples from the control monkeys shall remain free from rubella antibody.

2.2.4 Storage of virus seed lots

The freeze-dried virus seed lots shall be stored at or below -20°C; the liquid ones shall be stored at or below -60°C.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Lacto-albumin hydrolysate in MEM containing a quantity of inactivated calf serum can be used as the culture medium. Other suitable culture media can also be used. The quality of calf serum shall comply with the related requirements in Appendix III D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix III C).

2.3.4 Virus inoculation and cultivation

Incubate the cells at 37°C till confluent cell monolayer is formed. Inoculate the virus onto the cell cultures at an optimal MOI. Incubate the cultures at 30-32°C. When the CPE develop to a certain extent, flush the cell sheets with a rinsing solution, of which the volume in each vessel shall be not less than that of the original culture medium. MEM, medium 199 or other suitable media containing human blood albumin can be used as the maintenance medium.

2.3.5 Virus harvest

Harvest the virus suspension when the CPE reaches to a certain extent. After harvest, the culture, within an appropriate duration, may be further incubated for multiple harvests by replacement with fresh maintenance medium. The harvested virus suspension shall be kept frozen at or below -20°C .

2.3.6 Pooling

A number of single harvests derived from the same batch of cell cultures can be pooled into one batch of bulk.

2.3.7 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

The bulk can be properly diluted according to the virus titer. The final bulk is prepared by adding a quantity of stabilizer. A number of bulks can be pooled into one batch of final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

0.5 ml or 1.0 ml of the reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 3.2 lg CCID₅₀ of live rubella virus.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Virus titration

See Section 2.2.3.2. The titer shall be not less than 4.8 lg CCID₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content,

sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

See Section 2.2.3.1.

3.3.2 Inspection on final containers

The product looks like a milky-white crisp cake. After reconstitution, it shall turn into a clear, orange-red liquid, free of foreign matters.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Virus titration

See Section 2.2.3.2. A mixture of three to five containers of vaccine shall be made for virus titration. The titer shall be not less than 3.5 lg CCID₅₀/ml.

3.3.5 Thermostability test

Before release the final product shall be subject to thermostability test which shall be performed at the same time with virus titration in parallel. The vaccine samples that have been exposed at 37°C for 7 days shall be titrated following Section 2.2.3.2. The virus titer shall be not less than 3.5 lg CCID₅₀/ml. The loss of virus titer of the heat exposed vaccine shall be not more than 1.0 lg.

3.3.6 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at or below 8°C , protected from light. The validity period of rubella vaccine is 18 months starting from the date when the virus titration test proved qualified.

5 Package inserts

Directions for Use of Rubella Vaccine (Rabbit Kidney Cell), Live

[Drug name]

Adopted name: Rubella Vaccine (Rabbit Kidney Cell), Live

[Constituents and characters]

The vaccine is made from an attenuated rubella virus strain grown in primary rabbit kidney cell cultures. After cultivation, the virus suspension is harvested and an appropriate stabilizer is added for lyophilization. The vaccine looks like a milky-white crisp cake. After reconstitution, it shall turn into a clear, orange-red liquid.

[Eligibles]

Children susceptible to rubella at or above 8 months of age.

[Function and use]

The product can induce immunity against rubella virus in recipients following immunization. It is used to prevent rubella.

[Specifications]

0.5 ml or 1.0 ml of the reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 3.2 lg CCID₅₀ of live rubella virus.

[Administration and dosage]

(1) Reconstitute the vaccine with the stated amount of sterile water for injection. Shake the container till the content is reconstituted completely before use.

(2) Inject s.c. 0.5 ml of the vaccine at deltoid insertion area of the lateral upper arm.

[Adverse reactions]

Normally there are no local reactions at the inoculation site. A small number of recipients might have transient fever or mild skin rashes during the period between 6 and 11 days after injection. The reactions would not last longer than 2 days and could be relieved spontaneously. Some adult individuals may have mild arthralgia during the period of 2-4 weeks after injection. No particular treatment is necessary. Symptomatic treatment could be adopted in case of need.

[Contraindications]

- (1) Subjects with serious diseases or fever.
- (2) Those with a history of allergic reaction.
- (3) Women in pregnancy.

[Precautions]

- (1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.
- (2) Do not use the vaccine if any leakage of container or illegible label is found, or the content of the vaccine can not be completely reconstituted.
- (3) The vaccine should be kept at 2-8°C and used up within one hour after reconstitution; the remaining vaccine shall be discarded.
- (4) Women of childbearing age shall take some contraceptive measures for at least 3 months after immunization.
- (5) The immunization of rubella vaccine should be deferred for at least one month following administration of immunoglobulin.
- (6) Do not receive otherwise live vaccines one month before or after administering this vaccine. However, the vaccine can be given with live measles and mumps vaccines concomitantly.

[Storage and shipping]

Store and ship at 2-8°C, protected from light.

[Packaging]**[Validity period]**

18 months.

[Standard for implementation]**[Product license number]****[Manufacturer]**

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Mumps Vaccine, Live

Mumps vaccine is a preparation of live attenuated mumps virus grown in chick primary embryo cell culture. After cultivation and harvest, the virus suspension, after adding a suitable stabilizer, is lyophilized to make the vaccine. It is used to prevent mumps.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for the vaccine production

Primary chick embryo cells are used for the preparation of virus seeds and vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.2 Cell substrate preparation

The chick embryo of 9-11 days old from SPF stocks shall be selected and trypsinized to prepare dispersed cells which are then cultivated with an appropriate medium.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

Strain S₇₉ and strain Wm₈₄ or other approved attenuated mumps virus strains can be used as the viral seed for the vaccine production.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

The vaccine production shall be based on a seed lot system. The final product derived from Strain S₇₉ shall not exceed the 7th passage and the final product derived from strain Wm₈₄ shall not exceed the 11th passage.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Dilute the sample of seed virus to make a virus suspension at a concentration of 500-2000 CCID₅₀/ml. Mix the virus suspension with an equal volume of mumps antiserum. Keep the mixture in 37°C water bath for 60 minutes. Inoculate the incubated mixture onto FL or Vero cells. Evaluate the results after incubation for 8-10 days at a suitable temperature. Mumps virus shall be completely neutralized (no CPE observed); meanwhile, both the results of the serum control and the cell control shall be negative. The titer of reference virus preparation shall be not less than 500 CCID₅₀/ml.

2.2.3.2 Virus titration

Dilute the sample of virus seed 10-fold serially. Inoculate the virus suspensions at different dilutions onto FL or Vero cell cultures. The results are evaluated after incubation at a suitable temperature for 8-10 days. The titer shall be not less than 5.5 lg CCID₅₀/ml. Titration of the virus reference preparation shall be carried out in parallel.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix XIII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XIII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix XIII C).

2.2.3.6 Test for immunogenicity

Prepare the original vaccine with the master seed virus to immunize at least thirty healthy and susceptible children. Blood samples are taken for determination of mumps antibody before and 4-6 weeks after the inoculation, respectively. The antibody conversion rate shall be not less than 90% (HI and neutralization titer <1 : 2 as negative, HI and neutralization titer ≥1 : 2 as positive).

2.2.3.7 Test for neurovirulence in monkeys

The master seed lot or working seed lot shall be demonstrated to be free from neurovirulence. At least ten monkeys, serologically negative for mumps immediately prior to the neurovirulence test, shall be employed for each time. The test sample shall be given to each monkey by inoculation of 0.5 ml (representing not less than the virus content of one single human dose of vaccine) into the thalamic region of each hemisphere. The monkeys shall be observed for 17-21 days. No symptoms and signs of paralysis or any evidences of neurological involvement shall be found. Monkeys, if not more than two, that died

within 48 hours after injection may be replaced. The test is invalid and shall be repeated if more than 20% of the monkeys die even due to nonspecific causes. At the end of the observation period each monkey is bled and the individual sera shall be tested for mumps antibody. The seroconversion rate shall be not less than 80%. Each monkey is subjected to autopsy. Histo-pathological examinations of appropriate areas of the brain and spinal cord shall be made for any evidence of central nervous system involvement. The results shall be negative. Meanwhile, at least two mumps-susceptible monkeys shall be reserved as controls. Blood samples shall be taken from the control monkeys at the time of inoculating the test monkeys, and on the 10th day after the test animals are killed. All the serum samples from the control monkeys shall remain free from mumps antibody.

2.2.4 Storage of virus seed lots

The freeze-dried virus seed lots shall be stored at or below -20°C; the liquid ones shall be stored at or below -60°C.

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Earle solution containing lacto-albumin hydrolysate and a quantity of inactivated calf serum can be used as the culture medium. Other suitable culture media can also be used. The quality of calf serum shall comply with the related requirements in Appendix XIII D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix XIII C).

2.3.4 Virus inoculation and cultivation

The mixture of the virus and the dispersed cells at an optimal MOI shall be inoculated in the culture vessels which are then incubated at a suitable temperature. Discard the culture medium when the CPE develops to a certain extent. Flush the cell sheets with a rinsing solution of which the volume in each vessel shall be not less than that of the original culture medium. Maintenance medium is added after rinsing to continue the cultivation.

2.3.5 Virus harvest

Harvest the virus suspension when the CPE reaches to a certain extent. After harvest, the culture may be further incubated for multiple harvests by replacement with fresh maintenance medium.

2.3.6 Pooling

A number of single harvests derived from the cultures made of the same lot of dispersed cells can be pooled into one batch of bulk.

2.3.7 Control tests on bulk

See Section 3. 1.

2. 4 Final bulk

2. 4. 1 Formulation

The bulk can be properly diluted according to the virus titer. The final bulk shall be prepared by adding a quantity of stabilizer. A number of bulks can be pooled into one batch of final bulk.

2. 4. 2 Control tests on final bulk

See Section 3. 2.

2. 5 Final product

2. 5. 1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2. 5. 2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. During the filling process, the final bulk shall be kept cool in ice bath.

2. 5. 3 Specifications

0.5 ml or 1.0 ml of reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 3.7 lg CCID₅₀ of live mumps virus.

2. 5. 4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3. 1 Control tests on bulk

3. 1. 1 Virus titration

See Section 2. 2. 3. 2. The titer shall be not less than 5.0 lg CCID₅₀/ml.

3. 1. 2 Sterility test

It complies with the test for sterility (Appendix XIII A).

3. 1. 3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XIII B).

3. 2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3. 3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3. 3. 1 Identity test

See Section 2. 2. 3. 1.

3. 3. 2 Inspection on final containers

The product looks like a milky-white crisp cake. After reconstitution, it shall turn into a clear liquid, orange-red or pale pink in colour, free of foreign matters.

3. 3. 3 Moisture content

The residual moisture content shall be not more

than 3.0% (Appendix VII D).

3. 3. 4 Virus titration

See Section 2. 2. 3. 2. A mixture of three to five containers of vaccine shall be made for virus titration. The titer shall be not less than 4.0 lg CCID₅₀/ml.

3. 3. 5 Thermostability test

Before release the final product shall be subject to thermostability test which shall be performed at the same time with virus titration in parallel. The vaccine samples that have been exposed at 37°C for 7 days shall be titrated following Section 2. 2. 3. 2. The virus titer shall be not less than 4.0 lg CCID₅₀/ml. The loss of virus titer of the heat exposed vaccine shall be not more than 1.0 lg.

3. 3. 6 Content of residual bovine serum albumin

The residual bovine serum albumin content shall be not more than 50 ng/dose determined by ELISA.

3. 3. 7 Sterility test

It complies with the test for sterility (Appendix XIII A).

3. 3. 8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

4 Storage, shipping and validity period

Store and ship at or below 8°C, protected from light. The validity period of mumps vaccine is 18 months starting from the date when titration test proved qualified.

5 Package inserts

Directions for Use of Mumps Vaccine, Live

[Drug name]

Adopted name: Mumps Vaccine, Live

[Constituents and characters]

Mumps vaccine is a preparation of live attenuated mumps virus grown in chick primary embryo cell cultures. After cultivation and harvest, the virus suspension, after adding a suitable stabilizer, is lyophilized to make the vaccine. The vaccine looks like a milky-white crisp cake. After reconstitution, it turns into a clear liquid, orange-red or pale pink in colour.

[Eligibles]

Children susceptible to mumps at or above 8 months of age.

[Function and use]

The product can induce immunity against mumps virus in recipients following immunization. It is used to prevent mumps.

[Specifications]

0.5 ml or 1.0 ml of reconstituted vaccine per container. 0.5 ml per single human dose containing not less than 3.7 lg CCID₅₀ of live mumps virus.

[Administration and dosage]

(1) Reconstitute the vaccine with the stated amount of sterile water for injection. Shake the

container till the content is reconstituted completely before use.

(2) Inject s.c. 0.5 ml of the vaccine at deltoid insertion area of the lateral upper arm.

[Adverse reactions]

Normally there are no local reactions at the inoculation site. A small number of recipients might have transient fever or scattered skin rashes during the period between 6 and 10 days after injection. The reactions would not last longer than 2 days and could be relieved spontaneously. No particular treatment is necessary. Symptomatic treatment could be adopted in case of need.

[Contraindications]

(1) Subjects with serious diseases, acute or chronic infections or fever.

(2) Those with a history of allergic reaction to eggs.

(3) Women in pregnancy.

[Precautions]

(1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.

(2) Do not use the vaccine if any leakage of container or illegible label is found, or the content is not transparent after reconstitution.

(3) The vaccine should be kept at 2-8°C and used up within one hour after reconstitution; the remaining vaccine, if any, shall be discarded.

(4) The immunization of mumps vaccine should be deferred for at least one month following administration of immunoglobulin.

[Storage and shipping]

Store and ship at or below 8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Measles and Mumps Combined Vaccine, Live

Measles and mumps combined vaccine is a preparation made from live attenuated measles virus and mumps virus grown separately in chick embryo cell cultures. After cultivation and

harvest, the virus suspensions of the two viruses are pooled in proportion and lyophilized to make the combined vaccine after adding a suitable stabilizer. It is used to prevent measles and mumps.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall meet the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for the vaccine production

2.1.1 Cell substrates for measles vaccine production Primary chick embryo cells shall be used, which shall comply with the requirements in Section 2.1 of the Measles Vaccine, Live.

2.1.2 Cell substrates for mumps vaccine production Primary chick embryo cells shall be used and comply with the requirements in Section 2.1 of the Measles Vaccine, Live.

2.2 Virus seeds

2.2.1 Measles virus strains

Strain Hu-191 or other approved attenuated measles virus strains which comply with the Section 2.2 of the Measles Vaccine, Live can be used as the viral seed for the vaccine production.

2.2.2 Strain S₇₉ or other approved attenuated mumps virus strains which comply with the Section 2.2 of the Mumps Vaccine, Live can be used as the viral seed for the vaccine production.

2.3 Monovalent bulk

2.3.1 Preparation of bulk of measles vaccine

It complies with the Section 2.3 of the Measles Vaccine, Live.

2.3.2 Control tests on bulk of measles vaccine See Section 3.1.1.

2.3.3 Preparation of bulk of mumps vaccine

It complies with the Section 2.3 of the Mumps Vaccine, Live.

2.3.4 Control tests on bulk of mumps vaccine See Section 3.1.2.

2.4 Final bulk

2.4.1 Formulation

The monovalent bulk of measles and that of mumps vaccine qualified in related control tests are blended in a proportion, to which a quantity of stabilizer is added to formulate the final bulk.

2.4.2 Control tests on final bulk See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. During the filling process, the final bulk shall be kept cool in ice bath.

2.5.3 Specifications

0.5 ml of reconstituted vaccine per container.
0.5 ml per single human dose containing not less than 3.0 lg CCID₅₀ of live measles virus and not less than 3.7 lg CCID₅₀ of live mumps virus.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Control tests on bulk of measles vaccine

3.1.1.1 Identity test

Dilute the sample of the bulk of measles vaccine to make a virus suspension at a concentration of 500-2000 CCID₅₀/ml. Mix the virus suspension with an equal volume of measles antiserum and keep the mixture in 37°C water bath for 60 minutes. Inoculate the incubated mixture onto FL or Vero cells. Evaluate the results after incubation for 7-8 days at a suitable temperature. Measles virus shall be completely neutralized (no CPE observed); meanwhile, both the results of the serum control and the cell control shall be negative. The titer of reference virus preparation shall be not less than 500 CCID₅₀/ml.

3.1.1.2 Virus titration

Dilute the bulk sample 10-fold serially. Inoculate each dilution onto FL or Vero cell cultures. The result is evaluated after incubation at a suitable temperature for 7-8 days. The titer shall be not less than 4.8 lg CCID₅₀/ml. Titration of the virus reference preparation shall be conducted in parallel.

3.1.1.3 Sterility test

It complies with the test for sterility (Appendix III A).

3.1.1.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

3.1.2 Control tests on bulk of mumps vaccine

3.1.2.1 Identity test

Dilute the sample of the bulk of mumps vaccine to make a virus suspension at a concentration of 500-2000 CCID₅₀/ml. Mix the virus suspension with an equal volume of mumps antiserum and keep the mixture in 37°C water bath for 60 minutes. Inoculate the incubated mixture onto FL or Vero cells. Evaluate the result after incubation for 7-8 days at a suitable temperature. Mumps virus shall be completely neutralized (no CPE observed); meanwhile, both the results of the serum control and the cell control shall be negative. The titer of reference virus preparation shall be not less than

500 CCID₅₀/ml.

3.1.2.2 Virus titration

Dilute the bulk sample 10-fold serially. Inoculate each dilution onto FL cell or Vero cell cultures. The result is evaluated after incubation at a suitable temperature for 7-8 days. The titer shall be not less than 5.0 lg CCID₅₀/ml. Titration of the virus reference preparation shall be conducted in parallel.

3.1.2.3 Sterility test

It complies with the test for sterility (Appendix III A).

3.1.2.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix III A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Mix the properly diluted vaccine with the properly diluted anti-measles serum and anti-mumps serum. After incubation at 20-25°C for 90 minutes, inoculate the mixture onto Vero cell or FL cell cultures. The result is evaluated after incubation at 37°C for 7-8 days. The measles and mumps viruses shall be neutralized completely and no CPE shall be found. At the same time, serum and cell controls shall be set up, of which both the results shall be negative. The control of viruses shall be positive.

3.3.2 Inspection on final containers

The product looks like a milky-white crisp cake. After reconstitution, it turns into a clear, orange-red liquid, free of foreign matters.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Virus titration

A mixture of three to five containers shall be made for virus titration; the corresponding virus reference preparations shall be titrated in parallel. Measles vaccine virus titration; The measles virus is titrated in FL cell or Vero cell cultures after the mumps virus is neutralized with anti-mumps serum. The titer of measles virus shall be not less than 3.3 lg CCID₅₀/ml.

Mumps vaccine virus titration; The mumps virus is titrated in FL cell or Vero cell cultures after the measles virus is neutralized with anti-measles serum. The titer of mumps virus shall be not less than 4.0 lg CCID₅₀/ml.

3.3.5 Thermostability test

Before release the final product is subject to thermostability test, and the virus titration shall be carried out in parallel. The vaccine that has been exposed at 37°C for 7 days shall be titrated according to Section 3.3.4. The virus titer of measles vaccine shall be not less than 3.3 lg CCID₅₀/ml; the virus titers of mumps vaccine shall be not less than 4.0 lg CCID₅₀/ml. Both the losses of virus titers of these two vaccines shall be not more than 1.0 lg.

3.3.6 Content of residual bovine serum albumin

The residual bovine serum albumin content shall be not more than 50 ng/dose determined by ELISA.

3.3.7 Sterility test

It complies with the test for sterility (Appendix X A).

3.3.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix X F).

4 Storage, shipping and validity period

Store and ship at or below 8°C, protected from light. The validity period of the vaccine is 18 months starting from the date when the virus titration proved qualified.

5 Package inserts

Directions for Use of Measles and Mumps Combined Vaccine, Live

[Drug name]

Adopted name: Measles and Mumps Combined Vaccine, Live

[Constituents and characters]

Measles and mumps combined vaccine is a preparation made from live attenuated measles virus and mumps virus grown separately in chick embryo cell cultures. After cultivation and harvest, the virus suspensions of the two viruses are pooled in a proportion and lyophilized to make the combined vaccine after adding a suitable stabilizer. The product looks like a milky-white crisp cake. After reconstitution, it turns into a clear, orange-red liquid.

[Eligibles]

Subjects susceptible to measles and mumps at or above 8 months of age.

[Function and use]

The product can induce immunity against measles and mumps viruses in recipients following immunization. It is used to prevent measles and mumps.

[Specifications]

0.5 ml of reconstituted vaccine per container.
0.5 ml per single human dose containing not less than 3.0 lg CCID₅₀ of live measles virus and 3.7 lg CCID₅₀ of live mumps virus.

[Administration and dosage]

(1) Reconstitute the vaccine with the stated amount of sterile water for injection. Shake the container till the content is reconstituted completely before use.

(2) Inject s. c. 0.5 ml of the vaccine at deltoid insertion area of the lateral upper arm.

[Adverse reactions]

Normally there are no local reactions at the inoculation site. A small number of children recipients might have transient fever or scattered skin rashes during the period of 6-10 days after injection. The reactions would not last longer than 2 days and could be relieved spontaneously. Usually no particular treatment is necessary. Symptomatic treatment might be helpful in case of need.

[Contraindications]

(1) Subjects with serious diseases, acute or chronic infections.

(2) Those with fever.

(3) Those with a history of allergic reaction to eggs.

(4) Women in pregnancy.

[Precautions]

(1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.

(2) Do not use the vaccine if any leakage of container or illegible label is found, or the content is not transparent after reconstitution.

(3) The vaccine should be kept at 2-8°C and used up within one hour after reconstitution; the remaining vaccine, if any, shall be discarded.

(4) The immunization of measles and mumps combined vaccine should be deferred for at least one month following administration of immunoglobulin.

[Storage and shipping]

Store and ship at or below 8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Influenza Vaccine (Whole Virion), Inactivated

Influenza vaccine (whole virion) is a preparation



made from the prevalent strains of influenza virus type A and type B recommended by WHO and approved by the NRA. The strains of influenza virus type A and type B are grown separately in embryonated eggs. After cultivation and harvest, the virus suspension in allantoic cavity is inactivated, concentrated and purified to make the vaccine. It is used to prevent influenza.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Embryonated eggs for vaccine production

SPF chick embryos shall be used for the preparation and passage of virus seeds. Embryonated eggs from healthy flocks shall be used for the vaccine production. The embryos of 9-11 day old shall be in active status with vivid blood vessels and without any aberrations.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The virus seeds used for the vaccine production shall be the approved strains of influenza A and B viruses recommended by WHO, which shall be demonstrated by control test to be the prevalent virus strains or the similar ones.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics. The maximum number of passage of the seed lots shall not exceed the approved limit.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be conducted; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Hemagglutination-inhibition test shall be conducted with the corresponding subtype specific immune serum for typing identification of hemagglutinin. The result shall prove that the antigenicity of the test virus is in compliance with that of the recommended viral strain.

2.2.3.2 Virus hemagglutination (HA) test

The HA titer shall be not less than 1:120 determined by hemagglutination method.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix III A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

2.2.3.5 Tests for adventitious avian leucosis viruses

Neutralize the seed virus with the corresponding subtype specific antiserum. Inoculate the neutralized material to SPF chick embryo fibroblast cell cultures. After incubation, the cultures are tested by ELISA. The result shall be negative.

2.2.3.6 Tests for adventitious avian adenoviruses
Neutralize the seed virus with the corresponding subtype specific antiserum. Inoculate the neutralized material to SPF chick embryo hepatic cell cultures. After incubation, the cultures are tested for avian adenoviruses type I and type III by suitable serologic methods, respectively. All the results shall be negative.

2.2.4 Storage of virus seeds

Freeze-dried virus seeds shall be stored at or below -20°C , the liquid ones shall be stored at or below -60°C .

2.3 Monovalent bulk

2.3.1 Virus inoculation and cultivation

Inoculate a quantity of appropriately diluted virus from working seed lot into the allantoic cavities of chick embryos. Incubate the inoculated eggs at $33-35^{\circ}\text{C}$ for 48-72 hours. The remaining thawed seed virus, if any, is not allowed to freeze again for further use.

2.3.2 Virus harvest

After incubation, collect the living embryos and cool for a period of time at $2-8^{\circ}\text{C}$. Harvest the allantoic fluids in groups to make the single virus harvests.

2.3.3 Pooling

A number of single harvests from the embryos infected with monovalent virus seed can be pooled to make a monovalent virus pool.

2.3.4 Inactivation of monovalent virus pool

Add a quantity of formalin into the monovalent virus pool which is then kept at $2-8^{\circ}\text{C}$ for 7-10 days for inactivation.

2.3.5 Concentration and purification

2.3.5.1 Concentration

Concentrate the inactivated monovalent virus pool by ultrafiltration. The HA titer of the concentrated virus pool shall be not less than 1:10240.

2.3.5.2 Purification

Carry out the purification of the concentrated virus pool by column chromatography or sucrose density gradient zonal centrifugation. If the latter method is adopted, the sucrose shall be removed by means of ultrafiltration. The purified virus pool shall be sterilized by filtration to make a monovalent bulk.

2.3.6 Storage

Monovalent bulk shall be stored at $2-8^{\circ}\text{C}$.

2.3.7 Control tests on monovalent bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

The monovalent virus bulks of different types of virus can be pooled in proportion and properly diluted according to their respective hemagglutinin content. Thimerosal is added as a preservative to make the final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

0.5 ml or 1.0 ml per container. 0.5 ml or 1.0 ml per single human dose containing not less than 15 μg of hemagglutinin/virus strain.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on monovalent bulk

3.1.1 Identity test

Carry out the test of hemagglutination inhibition or radial immunodiffusion (RID) using the corresponding subtype of specific antiserum (See Section 3.1.3). The result shall prove that the antigenicity of the test sample is in compliance with that of the recommended strain.

3.1.2 Validation test for effective inactivation

Inoculate the virus suspensions at undiluted, 10^{-1} and 10^{-2} dilutions respectively into the allantoic cavity groups. Each group consists of ten chick embryos of 9-11 days old and the inoculum is 0.2 ml for each embryo. Incubate the inoculated eggs at 33-35°C for 72 hours. The embryos in that die within 24 hours after inoculation shall be excluded from evaluation. At least 80% of the embryos in each group shall survive the observation period. 0.5 ml of allantoic fluid from each surviving embryo in each group shall be taken and mixed for blind passage. Inoculate 0.2 ml of the mixed material from each group into each of another ten chick embryos. Incubate the eggs at 33-35°C for 72 hours. The allantoic fluid shall be sampled for test of hemagglutination test. The results of these three groups shall be negative (This test can also be conducted after inactivation of pooled monovalent virus bulk).

3.1.3 Hemagglutinin content

The determination of hemagglutinin content can be conducted by RID method. Add 10 μl of the reference antigen and test sample separately into the individual wells 3 mm in diameter on the 1.5% agarose gel plate containing reference antibody. Incubate the plates at 20-25°C for at least 18

hours. Then soak the plate in PBS for 1 hour followed by desiccation, staining and destaining. Measure precisely the diameters of the precipitation ring (A value) formed by the test samples. Figure out the linear regression equation by plotting the log of the concentration of the reference antigen with the log of its corresponding A value. The hemagglutinin content of the test sample is calculated by inserting the log A value into the linear regression equation. It shall be not less than 90 μg of each strain per ml.

3.1.4 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.2 Control tests on final bulk

3.2.1 Content of free formaldehyde

The content of free formaldehyde shall be not more than 50 μg /dose (Appendix VI L).

3.2.2 Thimerosal content

The thimerosal content shall be not more than 50 μg /dose (Appendix VII B).

3.2.3 Hemagglutinin content

See Section 3.1.3. It shall be not less than 15 μg /strain/dose.

3.2.4 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test

Carry out the RID test using the corresponding subtype specific antiserum. The results shall prove that the antigenicity of the test vaccine is in compliance with that of the recommended strains.

3.3.2 Inspection on final containers

The vaccine shall be a slightly milky-white liquid free of foreign matters.

3.3.3 Filling quantity

It complies with the requirements for filling (Appendix I A). The quantity shall be not less than the stated value.

3.3.4 Chemical tests

3.3.4.1 pH

The pH shall be 6.8-8.0 (Appendix V A).

3.3.4.2 Thimerosal content

The thimerosal content shall be not more than 50 μg /dose (Appendix VII B).

3.3.4.3 Total protein content

The total protein content shall be not more than 300 μg /dose (Appendix VI B, method 2) and shall not exceed 6 times of the HA content in the vaccine.

3.3.5 Hemagglutinin content

See Section 3.1.3. It shall be not less than 15 μg /strain/dose.

3.3.6 Content of ovalbumin



The content of ovalbumin shall be not more than 1000 ng/dose determined by ELISA (arbitration method) or counter immunoelectrophoresis method.

3.3.7 Test for bacterial endotoxin

The content of bacterial endotoxin shall be not more than 100 EU per human dose (Appendix XII E, the limit test of gel-clot method).

3.3.8 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period of the vaccine is 12 months starting from the date when the test for hemagglutinin content proved qualified.

5 Package inserts

Directions for Use of Influenza Vaccine (Whole Virion), Inactivated

[Drug name]

Adopted name: Influenza Vaccine (Whole Virion), Inactivated

[Constituents and characters]

Influenza vaccine (whole virion) is a preparation made from the prevalent strains or similar strains of influenza virus type A and type B grown separately in embryonated eggs. After incubation, the virus suspensions in allantoic cavities are harvested. The vaccine is prepared by inactivation, concentration and purification of the virus. It is a slightly milky-white liquid, containing thimerosal as a preservative.

[Eligibles]

Children aged above 12 years, adults and elderly persons.

[Function and use]

The product can induce immunity against influenza virus in recipients following immunization. It is used to prevent influenza.

[Specifications]

0.5 ml or 1.0 ml per container. 0.5 ml or 1.0 ml per single human dose containing not less than 15 µg of hemagglutinin/virus strain.

[Administration and dosage]

Inject i. m. the vaccine in the deltoid muscle of the lateral upper arm.

[Adverse reactions]

Erythema and swelling, pain, tenderness or itching at the injection site may appear in a few people between 12 and 24 hours after injection. Normally it does not last long or affect recipient's normal activity. A few recipients may manifest systemic reactions, such as myalgia, arthralgia, headache, malaise, and fever.

Anaphylactic reactions occur usually in the recipients with a history of allergy reaction to egg protein.

[Contraindications]

- (1) Subjects with fever, acute diseases or common cold.
- (2) Those with a history of Guillain-Barre syndrome.
- (3) Those with a history of allergy reaction to egg protein, or with other allergic conditions.
- (4) Women in pregnancy.

[Precautions]

- (1) Intravenous injection of the vaccine is strictly contraindicated!
- (2) Revaccination is prohibited if any neurological involvement occurs after injection.
- (3) Do not use the vaccine if any leakage of container or illegible label or any clumps not dispersed on shaking in the product is found.
- (4) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.
- (5) Freezing of the vaccine is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

12 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:
Address:
Zip code:
Tel:
Fax:
Web site:

Hepatitis B Vaccine Made by Recombinant DNA Techniques in Yeast

Recombinant hepatitis B vaccine in yeast is a preparation of purified hepatitis B surface antigen (HBsAg), expressed by the recombinant yeast. After purification of the HBsAg, an aluminum adjuvant is added to the purified HBsAg to make the vaccine. It is used to prevent hepatitis B.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Yeast seed for vaccine production

2.1.1 Name and origin of seed

The primary recombinant yeast seed No. 2150-2-3 (pHBS56-GAP347/33) expressing HBsAg obtained from Merck Sharp and Dohme Co., Inc. is used for the vaccine production. Other approved recombinant yeast seeds expressing HBsAg can also be used.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

The yeast seed provided by MSD is expanded by one subculture to prepare the master seed lot. Working seed lots are prepared by a single passage of expanding propagation removed from the master seed lot.

2.1.3 Control tests on seed lots

Control tests described below shall be fully implemented for both master and working seed lots.

2.1.3.1 Test for the purity of cultures

Inoculate the culture sample onto Columbia blood agar plate and enzymatic soya protein agar plate. Incubate at 20-25°C and 30-35°C separately for 5-7 days. No bacteria or other fungi shall be detected.

2.1.3.2 Test for plasmid retaining rate

The retaining rate of the plasmids shall be not less than 95% by replica plating technique.

2.1.4 Storage of yeast seeds

The master and working seed lots shall be preserved in liquid nitrogen. The storage period of working seed lots in -70°C freezer shall not exceed 6 months.

2.2 Bulk

2.2.1 Fermentation

The working seed lot is subject to three-step fermentation, i.e. cultivations in Erlenmeyer flasks, bioreactor and fermentor successively at a suitable temperature for a certain period of time. The harvested yeast cells shall be kept frozen.

2.2.2 Control tests on fermented products

2.2.2.1 Test for the purity of cultures

See Section 2.1.3.1.

2.2.2.2 Test for plasmid retaining rate

The retaining rate of the plasmids shall be not less than 90% by replica plating technique.

2.2.3 Purification

Cell disruptor is used to crush the yeast cells. Remove the cell debris. Conduct preliminary purification of HBsAg by means of silica gel adsorption followed by hydrophobic chromatography for further purification. After thiocyanate treatment the product is diluted and sterilized by filtration.

2.2.4 Control tests on purified product

2.2.4.1 Sterility test

It complies with the test for sterility (Appendix VII A).

2.2.4.2 Protein content

The protein content shall be 20.0-27.0 µg/ml or 60.0-81.0 µg/ml (Appendix VI B, method 2).

2.2.4.3 Specific protein band

Carry out the test for specific protein band (Appendix IV C). The molecular weight shall be within the range of 20-25 kD.

2.2.4.4 Purity

Carry out the test for purity by immunoblot (Appendix VIII A). The sample shall not show any irrelevant protein bands of yeast protein other than that accepted by the NCL.

Carry out the test for purity by HPLC method (Appendix III B). The content of HBsAg shall be not less than 99.0%; or the content of contaminated protein shall be not more than 1.0%.

2.2.4.5 Content of bacterial endotoxin

It shall be less than 10 EU per human dose (Appendix VIII E, the limit test of gel-clot method).

2.2.5 Bulk

2.2.5.1 Formulation

A quantity of formalin is added to the purified product, and kept at 37°C for a certain period of time. The bulk is then made by adding aluminum hydroxide for adsorption and thimerosal as a preservative.

2.2.5.2 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

The final bulk is made by blending the bulk in which the protein content is 20.0-27.0 µg/ml or 60.0-81.0 µg/ml with an equal volume of aluminum adjuvant or in the proportion of 1 : 5.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

0.5 ml or 1.0 ml per container. 0.5 ml per single human dose containing 5 µg of HBsAg or 1.0 ml per single human dose containing 10 µg of HBsAg.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Test for completeness of adsorption

After adsorption with aluminum hydroxide, the

test sample shall be centrifuged (6500 g) for 5 minutes. The HBsAg contents in the reference preparation, the test sample and the supernatant of the test sample shall be determined according to Appendix X A. The linear regression equation is obtained by plotting the log of the HBsAg concentration of the reference against the log of its absorbance value. The correlation coefficient of the regression equation shall be not less than 0.99. The contents of HBsAg can be figured out by using the regression equation with the absorbance values of the sample and the supernatant. The rate of adsorption can be calculated according to the following formula, and shall be not less than 95%.

$$P = \left(1 - \frac{C_s}{C_t}\right) \times 100\%$$

Where P = rate of adsorption (%)

C_s = HBsAg content in the supernatant of test sample ($\mu\text{g/ml}$);

C_t = HBsAg content in the test sample ($\mu\text{g/ml}$).

3.1.2 Thiocyanate content

Take the test sample before thimerosal is added. Centrifuge (6500 g) for 5 minutes and collect the supernatant. The following reagents shall be successively added into the test tubes in duplicate: thiocyanate standard solutions (1.0 μg , 2.5 μg , 5.0 μg and 10 $\mu\text{g/ml}$), the supernatant of test sample, and physiological saline, and the volume of each reagent shall be 5.0 ml. Then into each tube add 0.5 ml of borate buffer (pH 9.2), 0.5 ml of 2.25% chloramines T-physiological saline and 1.0 ml of 50% pyridine solution (formulated with physiological saline) successively. After addition of each reagent, the tube shall be shaken immediately to get the content homogenized. Keep the tubes standing for 10 minutes when all the above reagents have been added. Determination of the absorbance of each tube shall be carried out at a wavelength of 415 nm against physiological saline as the blank control. The linear regression equation is obtained by plotting the content of the thiocyanate in the standard against its average absorbance to figure out the correlation coefficient which shall be not less than 0.99. The content of the thiocyanate is calculated by using the regression equation with the average absorbance of the supernatant of the test sample. It shall be less than 1.0 $\mu\text{g/ml}$.

3.1.3 Content of Triton X-100

Take the test sample before thimerosal is added. Centrifuge (6500 g) for 5 minutes and collect. The following reagents shall be successively added into the test tubes in duplicate: Triton X-100 standard solutions (5 μg , 10 μg , 20 μg , 30 μg and 40 $\mu\text{g/ml}$), the supernatant of test sample and physiological saline, and the volume of each reagent shall be 2.0 ml. Add 1.0 ml of 5% (V/V) phenol solution into each tube and shake the tubes

immediately to get the content homogenized. Keep the tubes standing for 15 minutes at room temperature. Determination of the absorbance of each tube shall be carried out at a wavelength of 340 nm against physiological saline as the blank control. The linear regression equation is obtained by plotting the content of Triton X-100 in the standard against its average absorbance to figure out the correlation coefficient which shall be not less than 0.99. The content of Triton X-100 is calculated by using the regression equation with the average absorbance of the supernatant of the test sample. It shall be less than 15.0 $\mu\text{g/ml}$.

3.2 Control tests on final bulk

3.2.1 Chemical tests

3.2.1.1 pH

The pH shall be 5.5-7.2 (Appendix V A).

3.2.1.2 Content of free formaldehyde

It shall be less than 20 $\mu\text{g/ml}$ (Appendix VI L).

3.2.1.3 Aluminum content

The aluminum content shall be 0.35-0.62 mg/ml (Appendix VII F).

3.2.1.4 Thimerosal content

The thimerosal content shall be 30-70 $\mu\text{g/ml}$ (Appendix VII B).

3.2.1.5 Osmolarity

The osmolarity shall be 280 ± 65 mOsmol/L (Appendix V H).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.3 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/ml (Appendix XIII E, the limit test of gel-clot method).

3.3 Control tests on final product

3.3.1 Identity test

The HBsAg shall be identified by ELISA.

3.3.2 Inspection on final containers

The product is a milky-white suspension. Stratified precipitates may form which can be dispersed by shaking. No clumps shall be found on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 5.5-7.2 (Appendix V A).

3.3.3.2 Aluminum content

The aluminum content shall be 0.35-0.62 mg/ml (Appendix VII F).

3.3.3.3 Thimerosal content

The thimerosal content shall be 30-70 $\mu\text{g/ml}$ (Appendix VII B).

3.3.4 Relative potency

The relative potency *in vitro* shall be not less than 0.5 (Appendix X A).

3.3.5 Sterility test

It complies with the test for sterility (Appendix III A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix III F).

3.3.7 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/ml (Appendix III E, the limit test of gel-clot method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period of the vaccine is 24 months starting from the date when the potency test proved qualified.

5 Package inserts

Directions for Use of Hepatitis B Vaccine Made by Recombinant DNA Techniques in Yeast

[Drug name]

Adopted name: Hepatitis B Vaccine Made by Recombinant DNA Techniques in Yeast

[Constituents and characters]

The vaccine is a preparation of purified hepatitis B surface antigen (HBsAg), expressed by the recombinant yeast. After purification an aluminum adjuvant is added to the purified HBsAg for adsorption. The vaccine is a milky-white suspension with a slight precipitate which can be dispersed on shaking. It contains thimerosal as a preservative.

[Eligibles]

The subjects susceptible to hepatitis B virus, particularly for the following:

- (1) Newborns, especially those whose mothers are HBsAg and/or HBeAg positive;
- (2) The health care personnel or the laboratory workers handling human blood.

[Function and use]

The product can induce immunity against hepatitis B virus in recipients following immunization. It is used to prevent hepatitis B.

[Specifications]

0.5 ml or 1.0 ml per container. 0.5 ml per single human dose containing 5 µg of HBsAg or 1.0 ml per single human dose containing 10 µg of HBsAg.

[Administration and dosage]

- (1) Inject i. m. the vaccine in the deltoid muscle of the lateral upper arm.
- (2) Three injections are required for a complete immunization course at the schedule of 0, 1 and 6 months. The first injection for the newborns shall be given within 24 hours after birth. For the eligibles under 16 years of age, each single human dose contains 5 µg of HBsAg, and 10 µg for those above 16 years of age.

[Adverse reactions]

Erythema and swelling and pain at the site of

injection or low fever may appear in a few people. Normally no treatment is necessary. It could be relieved spontaneously. Systemic treatment could be adopted in case of need.

[Contraindications]

- (1) Subjects with fever, acute disease or serious chronic diseases;
- (2) Those with a history of allergic reaction to yeast.

[Precautions]

- (1) Shake the container well before use.
- (2) Do not use the vaccine if any leakage of container or clumps not dispersed on shaking is found.
- (3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.
- (4) Freezing of the vaccine is contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Hepatitis B Vaccine Made by Recombinant DNA Techniques in CHO Cell

Recombinant hepatitis B vaccine in CHO cells is a preparation of purified hepatitis B surface antigen (HBsAg), expressed by the recombinant CHO cells. After purification of the HBsAg, a quantity of aluminum hydroxide is added as an adjuvant to make the vaccine. It is used to prevent hepatitis B.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

2.1.1 Name and origin of cell strain

The CHO cell strain C₂₈ expressing HBsAg obtained



by recombinant DNA techniques is used for the vaccine production.

2.1.2 Establishment of cell banks

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

The master cell bank shall not exceed the 21th passage; the working cell bank shall not exceed the 26th passage; the cells used in vaccine production shall not exceed the 34th passage.

2.1.3 Control tests on cell banks

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3.1 Tests for adventitious viruses

The tests for bacteria and fungi, mycoplasmas and adventitious viruses shall comply with the requirements.

2.1.3.2 Identity test

Any approaches such as isoenzyme analysis, biochemical tests, immunological, cytological as well as the genetic marker tests can be used for the identification of the typical CHO cell.

2.1.3.3 Expressed quantity of HBsAg

The expressing level of HBsAg in the cells from master and working cell banks shall be not less than that of the primary cell bank.

2.1.4 Storage of cell seeds

The cell seeds shall be stored in a liquid nitrogen.

2.2 Bulk

2.2.1 Cell substrate preparation

Cell cultures are dispersed by trypsinization and cultivated under an optimum condition.

2.2.2 Culture medium

DMEM containing a quantity of inactivated calf serum can be used as the culture medium. The quality of calf serum shall comply with the requirements in Appendix VIII D.

2.2.3 Harvest

Harvest the culture supernatant when the expressed HBsAg content has reached over 1.0 mg/L.

2.2.4 Purification

Purification of HBsAg should be carried out using the approval method.

2.2.5 Formaldehyde treatment

A quantity of formalin is added to the purified HBsAg in a proportion of 1 : 2000. Keep the treated HBsAg at 37°C for 72 hours.

2.2.6 Sterilization by filtration

After ultrafiltration, concentration and sterilization by filtration, the treated HBsAg is defined as the bulk (the process of sterilization by filtration can also be carried out before formaldehyde treatment).

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

Dilute the bulk according to its protein content to make a final concentration of HBsAg of 10 µg/ml or 20 µg/ml. Add aluminum hydroxide for adsorption and thimerosal as a preservative to make the final bulk.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

1.0 ml per container, 1.0 ml per single human dose containing 10 µg or 20 µg of HBsAg.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

The protein content shall be 100-200 µg/ml (Appendix VI B, method 2).

3.1.2 Specific protein bands

Carry out the test for specific protein bands (Appendix IV C). The protein bands of 23 kD and 27 kD shall be shown in the separation gel, and the protein band of 30 kD and the band of dimer of HBsAg may also be shown.

3.1.3 Purity

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method: Size exclusion chromatographic column filled with hydrophilic resin is used. Exclusion limit is lower than 1000 kD, porosity is 100 nm, granularity is 17 µm, internal diameter is 7.5 mm and column length is 30 cm. Flow phase is 0.05 mol/L phosphate buffer of pH 6.8. The detecting wavelength is 280 nm. The purity of HBsAg shall be not less than 95.0% calculated based on the area normalization method.

3.1.4 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.1.5 Residual DNA content of CHO cells

The residual DNA content of CHO cells shall be not more than 10 pg/ml (Appendix IX B).

3.1.6 Content of residual CHO cell proteins

The content of residual CHO cell proteins shall be not more than 0.05% of the total protein content

determined by immunoenzymometric assay.

3.1.7 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.1.8 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XIII B).

3.2 Control tests on final bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.2.2 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU per human dose (Appendix XIII E, the limit test of gel-clot method).

3.3 Control tests on final product

3.3.1 Identity test

The HBsAg shall be identified by ELISA.

3.3.2 Inspection on final containers

The product is a milky-white suspension. Stratified precipitates may form which can be dispersed on shaking. No clumps shall be found on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 5.5-6.8 (Appendix V A).

3.3.3.2 Aluminum content

The aluminum content shall be not more than 0.43 mg/ml (Appendix VII F).

3.3.3.3 Thimerosal content

The thimerosal content shall be not more than 100 µg/ml (Appendix VII B).

3.3.3.4 Content of free formaldehyde

It shall be not more than 50 µg/ml (Appendix VI L).

3.3.4 Potency test

Dilute the sample of test vaccine serially. Inoculate 1.0 ml of each dilution i. p. into each of twenty NIH mice or BALB/c mice (female, non-pregnant) of 4-5 weeks old. The reference vaccine shall be tested in parallel as a control. Animals are bled 4-6 weeks after inoculation. Determine the anti-HBs by RIA or ELISA and calculate the ED₅₀. The ratio of the ED₅₀ of the test vaccine to the ED₅₀ of reference vaccine shall be not less than 1.0.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

3.3.7 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU per human dose (Appendix XIII E, the limit test of gel-clot method).

4 Storage and validity period

Store and ship at 2-8°C, protected from light. The validity period of the vaccine is 24 months starting from the date when the potency test proved qualified.

5 Package inserts

Directions for Use of Hepatitis B Vaccine Made by Recombinant DNA Techniques in CHO Cell

[Drug name]

Adopted name: Hepatitis B Vaccine Made by Recombinant DNA Techniques in CHO Cell

[Constituents and characters]

Recombinant hepatitis B vaccine in CHO cells is a preparation of purified hepatitis B surface antigen (HBsAg), expressed by the recombinant CHO cells. After purification of the HBsAg, a quantity of aluminum hydroxide is added to make the vaccine. The vaccine is a milky-white suspension with a slight precipitate which can be dispersed on shaking. It contains thimerosal as a preservative.

[Eligibles]

The subjects susceptible to hepatitis B virus, particularly for the following:

- (1) Newborns, especially those whose mothers are HBsAg and/or HBeAg positive.
- (2) The medical and nursing staffs or the laboratory workers handling human blood.

[Function and use]

The product can induce immunity against hepatitis B virus in recipients following immunization. It is used to prevent hepatitis B.

[Specifications]

1.0 ml per container. 1.0 ml per single human dose containing 10 µg or 20 µg of HBsAg.

[Administration and dosage]

- (1) Inject i. m. the vaccine in the deltoid muscle of the lateral upper arm.
- (2) Three injections are required for a complete immunization course at the schedule of 0, 1 and 6 months. For newborns the first injection shall be given within 24 hours after birth. For the susceptible subjects, each single human dose contains 10 µg of HBsAg and for the newborns whose mothers are HBsAg and/or HBeAg positive, each single human dose contains 20 µg of HBsAg.

[Adverse reactions]

Erythema and swelling and pain at the site of injection or low fever may appear in a few people. Normally no treatment is necessary. It could be relieved spontaneously. Systemic treatment could be adopted in case of need.

[Contraindications]

- (1) Subjects with fever, acute or serious chronic diseases.
- (2) Those with a history of allergy.

[Precautions]

- (1) Shake the vaccine container well before use.



(2) Do not use the vaccine, if any leakages of the container or clumps not dispersed on shaking are found.

(3) The recipients shall take a rest for a while on site following immunization. Adrenaline should be available for first aid in case of severe anaphylactic reactions.

(4) Freezing of the vaccine is strictly contraindicated.

[Storage]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Hepatitis A Vaccine, Live

The hepatitis A vaccine is a liquid preparation of the live attenuated strain of hepatitis A virus (HAV) grown in human diploid cells. After cultivation and harvest, the virus suspension is purified to make the vaccine. It is used to prevent hepatitis A.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for production

Human diploid cells (strain KMB₁₇ or strain 2BS) can be used for the vaccine production.

2.1.1 Management and control tests on cell banks

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

The cell cultures derived from the cells of one or several ampoules from the same lot of working cell bank through resurrection and propagation shall only be used for the production of one lot of vaccine.

For strain KMB₁₇, the maximum number of population doublings for the primary cell bank shall be the 6th; for the master cell bank be the 15th, for the working cell bank be the 45th.

For strain 2BS, the maximum number of population

doublings for the primary cell bank shall be the 14th; for the master cell bank be the 31st; for the working cell bank be the 44th.

2.1.2 Cell substrate preparation

Cells in one or several ampoules taken from working cell bank are resurrected and mixed to grow into monolayer. After digestion with a suitable concentration of trypsin, the dispersed cells can be incubated stationarily or by using roller bottles at 37°C ± 0.5°C.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The HAV attenuated strain H₂ or L-A-1 can be used as the seed for vaccine production.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

The vaccine production shall be based on a seed lot system. The final product shall not exceed the 15th passage for the virus strain H₂, and shall not exceed the 27th passage for the virus strain L-A-1.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Mix specific anti-HA serum with high titer and HA-negative serum with an equal volume of HA virus (500-1000 CCID₅₀/ml), respectively. Keep the virus-serum mixtures in 37°C water bath for 60 minutes. Inoculate the incubated mixtures onto the monolayers of human diploid cells. Incubate the cultures at 35°C till the virus replicates to its peak, then harvest and extract the HAV with routine process. Determine the HA virus by ELISA, it shall be completely neutralized and the virus mixed with HA-negative serum can be detected.

2.2.3.2 Virus titration

The sample of seed virus shall be diluted 10-fold serially. Inoculate at least three consecutive dilutions of the virus suspensions into culture tubes containing human diploid cells. Incubate at 35°C till the virus replicates to its peak. Collect and release the virus after harvest, and determine its titer by ELISA. The HAV titer shall be not less than 6.50 lg CCID₅₀/ml.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix III A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix III B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses

(Appendix XIII C).

2.2.3.6 Test for immunogenicity

The original vaccine prepared from the virus seed of the master seed lot is subject to the test. Immunize a group of at least forty HA-susceptible persons with the vaccine following routine practice. Take blood samples from the recipients before and 6-8 weeks after inoculation. The seroconversion rate shall be not less than 90%.

2.2.3.7 Tests for safety and immunogenicity in monkeys

The vaccine produced by the master seed virus is subject to the tests. Inoculate 1.0 ml of sample of the vaccine with a titer not less than $6.50 \lg \text{CCID}_{50}/\text{ml}$ into the vein of the lower limb of each of five healthy Macaca rhesus or stump-tailed monkeys (HA antibody negative, with normal ALT value, weighing 1.5-4.5 kg). Liver punctures for histopathological examination shall be applied to each monkey in 0, the 4th and the 8th weeks respectively after immunization. Blood samples shall be taken from each monkey in 0, the 2nd, the 3rd, the 4th, the 6th, and the 8th weeks respectively for the determination of ALT and HA antibody. Two healthy monkeys shall be reserved as negative control.

The test sample passes the tests if the results comply with the following criteria.

- (1) Seroconversion occurs in at least four monkeys.
- (2) No more than two monkeys can show a transient elevation of ALT value that occurs only in one week during the observation period.
- (3) No pathological changes in liver are related to the inoculated sample.

Retest is permitted if one of the following conditions occurs:

- (1) Seroconversion happens in less than four of the five inoculated monkeys.
- (2) Abnormal increases of ALT value more than 2 times are shown before and within 2 weeks after seroconversion.
- (3) Pathological changes in liver occur in the test monkeys, but irrelevant causes can not be excluded.

The vaccine shall be judged as unqualified if any one of the above mentioned conditions appear again in the retest.

2.2.4 Storage of virus seed lots

The virus seed lots shall be stored at or below -60°C .

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

MEM containing a quantity of inactivated newborn calf serum shall be used as the culture medium. Other suitable media can also be used. The quality of calf serum shall comply with the requirements in Appendix XIII D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix XIII C).

2.3.4 Virus inoculation, release and incubation
Inoculate the working seed virus at an optimal MOI onto the human diploid cell cultures. Incubate the virus-inoculated cultures at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ till the virus replicates to its peak. Replace the culture medium with maintenance medium after flushing the cell sheets with enough rinsing solution.

2.3.5 Virus harvest

Harvest the cultured suspension containing the supernatant and HAV infected cells. Treat the suspension with ultrasonic wave and/or several cycles of freezing and thawing. Purify the virus by means of chloroform extraction.

2.3.6 Pooling

After purification a number of single harvests derived from the same batch of cell cultures can be pooled into one batch of bulk under a strictly aseptic condition.

2.3.7 Control tests

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

The bulk can be properly diluted according to its virus titer. A quantity of stabilizer is added to make the final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1.0 ml per container. 1.0 ml per single human dose containing not less than $6.50 \lg \text{CCID}_{50}$ of live HA virus.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Virus titration

See Section 2.2.3.2. The virus titer of the bulk shall be not less than $7.00 \lg \text{CCID}_{50}/\text{ml}$.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.1.3 Test for mycoplasmas



It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control tests on final bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.2 Content of residual chloroform

The content of residual chloroform shall be not more than 0.1 % (Appendix VI O).

3.3 Control tests on final product

3.3.1 Identity test

The test shall be conducted by ELISA and the HAV antigen shall be demonstrated.

3.3.2 Inspection on final containers

The product is a clear liquid free of foreign matters.

3.3.3 Virus titration

See Section 2.2.3.2. A mixture of three to five containers of vaccine shall be made for virus titration. The virus titer shall be not less than 6.50 lg CCID₅₀/ml.

3.3.4 Thermostability test

Before release the final product shall be subject to thermostability test and the test shall be performed at the same time with virus titration in parallel. The vaccine samples that have been exposed at 37°C for 48 hours shall be titrated following Section 2.2.3.2. The loss of titer of the heat exposed vaccine shall be not more than 0.50 lg.

3.3.5 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.3.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period of the vaccine is 5 months starting from the date when the virus titration proved qualified.

5 Package inserts

Directions for Use of Hepatitis A Vaccine, Live

[Drug name]

Adopted name: Hepatitis A Vaccine, Live

[Constituents and characters]

The hepatitis A vaccine is a liquid preparation of the live attenuated strain of hepatitis A virus (HAV) grown in human diploid cells. After cultivation and harvest, the virus suspension is purified to make the vaccine. It is a clear liquid.

[Eligibles]

Hepatitis A susceptible individuals above the age of 18 months.

[Function and use]

The product can induce immunity against hepatitis A virus in recipients following immunization. It is used to prevent hepatitis A.

[Specifications]

1.0 ml of reconstituted vaccine per container.

1.0 ml per single human dose containing not less than 6.50 lg CCID₅₀ of live hepatitis A virus.

[Administration and dosage]

Inject 1.0 ml s.c. the vaccine at deltoid insertion area of the lateral upper arm.

[Adverse reactions]

A few recipients might have pain, erythema and swelling at the injection site, which could generally be relieved spontaneously within 3 days. Occasionally skin rash may appear and no special treatment is necessary. Symptomatic treatment could be helpful in case of need.

[Contraindications]

(1) Subjects with discomfort, body temperature (subaxillary) over 37.5°C.

(2) Those with acute infectious disease or other serious diseases.

(3) Those with immunodeficiency or receiving immunosuppressant.

(4) Those with a history of allergy.

[Precautions]

(1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.

(2) Do not use the vaccine if any leakage of container, foreign matters, or turbidity of content is found.

(3) The immunization of hepatitis A vaccine should be deferred for at least one month following administration of immunoglobulin.

(4) Use the vaccine cautiously for pregnant women.

(5) Freezing of vaccine is contraindicated.

[Storage and shipping]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

5 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Hepatitis A (Live) Vaccine, Freeze-dried

The hepatitis A vaccine is a freeze-dried preparation of the live attenuated strain of hepatitis A virus (HAV) grown in human diploid cell cultures. After cultivation and harvest, the virus suspension is purified and lyophilized after adding an appropriate stabilizer to make the vaccine. It is used to prevent hepatitis A.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Human diploid cells (strain KMB₁₇ or strain 2BS) can be used for the vaccine production.

2.1.1 Management and control tests on cell banks

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

The cell cultures derived from the cells of one or several ampoules from the same lot of working cell bank through resurrection and propagation shall only be used for the production of one batch of vaccine.

For strain KMB₁₇, the maximum number of population doublings for the primary cell bank shall be the 6th; for the master cell bank be the 15th, for the working cell bank be the 45th.

For strain 2BS, the maximum number of population doublings for the primary cell bank shall be the 14th; for the master cell bank be the 31st; for the working cell bank be the 44th.

2.1.2 Cell substrate preparation

Cells in one or several ampoules taken from working cell bank are resurrected and mixed to grow into monolayer. After digestion with a suitable concentration of trypsin, the dispersed cells can be incubated stationarily or by using roller bottles at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The HAV attenuated strain H₂ or L-A-1 can be used as the seed for vaccine production.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

For strain H₂, the passage of the primary seed lot shall not exceed the 7th; that of the master seed lot not exceed the 8th; and that of the working

seed lot not exceed the 14th. For strain L-A-1, the passage of the primary seed lot shall not exceed the 17th; that of the master seed lot not exceed the 18th; and that of the working seed lot not exceed the 26th.

2.2.3 Control tests on virus seed lots

For master seed lot, comprehensive control tests described below shall be carried out; for working seed lot, tests described in Sections 2.2.3.1-2.2.3.4 shall be carried out at least.

2.2.3.1 Identity test

Mix specific anti-HA serum with high titer and HA negative serum with an equal volume of HA virus (500-1000 CCID₅₀/ml), respectively. Keep the virus-serum mixtures in 37°C water bath for 60 minutes. Inoculate the incubated mixtures onto the monolayers of human diploid cells. Incubate the cultures at 35°C till the virus replicates to its peak, then harvest and extract the HAV with routine process. Determine the HA virus by ELISA, it shall be completely neutralized and the virus mixed with HA negative serum can be detected.

2.2.3.2 Virus titration

The sample of seed virus shall be diluted 10-fold serially. Inoculate at least three consecutive dilutions of the virus suspensions into tubes containing human diploid cells. Incubate at 35°C till the virus replicates to its peak. Collect and release the virus after harvest, and determine its titer by ELISA. The HAV titer shall be not less than 6.50 lg CCID₅₀/ml.

2.2.3.3 Sterility test

It complies with the test for sterility (Appendix XII A).

2.2.3.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

2.2.3.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix XII C).

2.2.3.6 Test for immunogenicity

The original vaccine prepared from the virus seed of the master seed lot is subject to the test. Immunize a group of at least forty HA-susceptible persons with the vaccine following routine practice. Take blood samples from the recipients before and 6-8 weeks after inoculation. The seroconversion rate shall be not less than 90%.

2.2.3.7 Tests for safety and immunogenicity in monkeys

The vaccine produced by the master seed virus is subject to the tests. Inoculate 1.0 ml of sample of the vaccine with a titer not less than 6.50 lg CCID₅₀/ml into the vein of the lower limb of each of five healthy Macaca rhesus or stump-tailed monkeys (HA antibody negative, with normal ALT value, weighing 1.5-4.5 kg). Liver punctures

for histopathological examination shall be applied to each monkey in 0, the 4th and the 8th weeks respectively after immunization. Blood samples shall be taken from each monkey in 0, the 2nd, the 3rd, the 4th, the 6th, and the 8th weeks respectively for the determination of ALT and HA antibody. Two healthy monkeys shall be reserved as negative control.

The test sample passes the tests if the results comply with the following criteria:

(1) Seroconversion occurs in at least four monkeys.

(2) No more than two monkeys can show a transient elevation of ALT value that occurs only in one week during the observation period.

(3) No pathological changes in liver are related to the inoculated sample.

Retest is permitted if one of the following conditions occurs.

(1) Seroconversion happens in less than four of the five inoculated monkeys.

(2) Abnormal elevation of ALT value more than two times are shown within two weeks before and after seroconversion.

(3) Pathological changes in liver occur in the test monkeys, but irrelevant causes can not be excluded.

The vaccine shall be judged as unqualified if any one of the above mentioned conditions appears again in the retest.

2.2.4 Storage of virus seed lots

The virus seed lots shall be stored at or below -60°C .

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

MEM containing a quantity of inactivated newborn calf serum shall be used as the culture medium. Other suitable media can also be used. The quality of calf serum shall comply with the requirements in Appendix XIII D.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix XIII C).

2.3.4 Virus inoculation and incubation

Inoculate the working seed virus at an optimal MOI onto the human diploid cells. Incubate the virus-inoculated cultures at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ till the virus replicates to its peak. Replace the culture medium with maintenance medium after flushing the cell sheets with enough rinsing solution.

2.3.5 Virus harvest

Harvest the cultured suspension containing the supernatant and HAV infected cells. Treat the suspension with ultrasonic wave and/or several cycles of freezing and thawing. Purify the virus by means of chloroform extraction.

2.3.6 Pooling

After purification a number of single harvests derived from the same batch of cell cultures can be pooled into one batch of bulk under a strictly aseptic condition.

2.3.7 Control tests

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

The bulk can be properly diluted according to its virus titer. A quantity of stabilizer is added to make the final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1.0 ml of reconstituted vaccine per container.

1.0 ml per single human dose containing not less than 6.50 lg CCID₅₀ of live HA virus.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Virus titration

See Section 2.2.3.2. The virus titer of the bulk shall be not less than 7.00 lg CCID₅₀/ml.

3.1.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control test on final bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.2 Content of residual chloroform

The content of residual chloroform shall be not more than 0.1 % (Appendix VI O).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

The test shall be conducted by ELISA and the HAV antigen shall be demonstrated.

3.3.2 Inspection on final containers

It looks like a milky-white crisp cake. After reconstitution it turns into a clear liquid and free of foreign matters.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Virus titration

See Section 2.2.3.2. A mixture of three to five containers of vaccine shall be made for virus titration. The virus titer shall be not less than 6.50 lg CCID₅₀/ml.

3.3.5 Thermostability test

Before release the final product shall be subject to thermostability test and the test shall be performed at the same time with virus titration in parallel. The vaccine samples that have been exposed at 37°C for 72 hours shall be titrated following Section 2.2.3.2. The loss of titer of the heat exposed vaccine shall be not more than 0.50 lg.

3.3.6 Content of residual bovine serum albumin
The content of residual bovine serum albumin shall be not more than 50 ng/dose determined by ELISA.

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period of the vaccine is 18 months starting from the date when the virus titration proved qualified.

5 Package inserts

Directions for Use of Hepatitis A (Live) Vaccine, Freeze-dried

[Drug name]

Adopted name: Hepatitis A (Live) Vaccine, Freeze-dried

[Constituents and characters]

The freeze-dried hepatitis A vaccine is a preparation of the live attenuated strain of hepatitis A virus (HAV) grown in human diploid cell cultures. After cultivation and harvest, the virus suspension is purified, and lyophilized after adding an appropriate stabilizer to make the vaccine. It looks like a milky-white crisp cake; after reconstitution it turns into a clear liquid.

[Eligibles]

Hepatitis A susceptible individuals above the age of 18 months.

[Function and use]

The product can induce immunity against hepatitis A virus in recipients following immunization. It is used to prevent hepatitis A.

[Specifications]

1.0 ml of reconstituted vaccine per container.
1.0 ml per single human dose containing not less than 6.50 lg CCID₅₀ of live hepatitis A virus.

[Administration and dosage]

Reconstitute the vaccine with the stated amount of sterile water for injection. Shake the container till the content is reconstituted completely before use. Inject 1.0 ml s.c. the reconstituted vaccine at deltoid insertion area of the lateral upper arm.

[Adverse reactions]

A few recipients might have pain, erythema and swelling at the injection site, which could generally be relieved spontaneously within 3 days. Occasionally skin rash may appear and no special treatment is necessary. Symptomatic treatment could be helpful in case of need.

[Contraindications]

- (1) Subjects with discomfort, body temperature (subaxillary) over 37.5°C.
- (2) Those with acute infectious disease or other serious diseases.
- (3) Those with immunodeficiency or receiving immunosuppressant.
- (4) Those with a history of allergy.

[Precautions]

- (1) Care should be taken to avoid contacting the vaccine by disinfectant during opening the container and in the course of injection.
- (2) Do not use the vaccine if any leakage of container, foreign matters, or turbidity of content is found.
- (3) The immunization of hepatitis A vaccine should be deferred for at least one month following administration of immunoglobulin.
- (4) Use the vaccine cautiously for pregnant women.

[Storage and shipping]

Store and ship at 2-8°C, protected from light.

[Packaging]

[Validity period]

18 months.

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:
Address:
Zip code:
Tel:
Fax:
Web site:

Poliomyelitis Vaccine in Dragee Candy (Human Diploid Cell), Live

The poliomyelitis vaccine in dragee candy is a

preparation of live attenuated poliovirus (types I, II, and III) grown in human diploid cell cultures. After cultivation, the virus suspension is harvested and made into a dragee candy form. It is used to prevent poliomyelitis.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

Human diploid cell cultures shall be used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

The cell cultures derived from the cells of one or several ampoules from the same lot of working cell bank through resurrection and propagation shall only be used for the production of one lot of vaccine.

The maximum number of population doublings for the master cell seed bank shall be the 23rd; for the working cell bank shall be the 27th; and for the final product shall be the 44th.

2.1.2 Cell substrate preparation

The cells from the working cell bank are resurrected and expanded to produce a collection of cell cultures. The cell of monolayer is digested by 0.25% trypsin and the dispersed cells are further expanded with an appropriate split rate. Cultivate the cells at 37°C for anchorage in a stationary way or in a rolling bottles.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The virus strains used as the seeds for the vaccine production shall be Types I, II and III attenuated strains, such as Sabin strain (Types I, II and III), purified Sabin strain (Types I, II and III), Zhong III₂ strain or other approved strains.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.2.2.1 Primary seed lot

Strains of Sabin types I, II, III and Zhong III₂ were prepared and are preserved by the original researchers.

2.2.2.2 Master seed lot

A batch of virus suspension of uniform composition, which is prepared by subculture for one to two passages from the primary seed virus in fetal monkey kidney cells or human diploid cells,

is defined as the master seed lot.

The passage level of the Sabin master seed is SO+1. The passage level of Zhong III₂ master virus seed is Zhong III₂ 1. The master virus seed of type III Pfizer strain is RSO1.

2.2.2.3 Working seed lot

A batch of virus suspension of uniform composition, which is prepared from the master seed virus by subculture for one to two passages in human diploid cells, is defined as the working seed lot.

2.2.3 Virus passage

For the passages from the primary seed lot to the working seed lot, Sabin I, II and other purified strains as well as Zhong III₂ shall not exceed three passages; Sabin III and other purified strains including Pfizer strain shall not exceed two passages.

The cell cultures used for the preparation of virus seed lot for the production of vaccine shall be limited to fetal monkey kidney cells or human diploid cells.

2.2.4 Control tests on virus seed lots

For master and working seed lots, comprehensive control tests described below shall be conducted, unless otherwise specified.

2.2.4.1 Identity test

A quantity of the virus sample is mixed with an equal volume of monovalent antiserum against poliovirus type I, type II or type III correspondingly. After incubation at 35-37°C for 1-2 hours, the incubated mixture is inoculated onto Hep-2 or other susceptible cells. Read the results following incubation at 35-36°C for 7 days after inoculation. The virus type shall be identified serologically without any suspicions. The corresponding serum control and cell control shall be set up in parallel and their results shall be negative. The result of virus control must be positive.

2.2.4.2 Virus titration

Micro-titration method is adopted. Samples shall be diluted 10-fold serially. Inoculate virus suspensions at different dilutions onto Hep-2 or other susceptible cells. Incubate the inoculated cell cultures at 35-36°C for 7 days. The titer shall be not less than 6.5 lg CCID₅₀/ml. During virus titration the virus reference shall be titrated in parallel.

2.2.4.3 Sterility test

It complies with the test for sterility (Appendix VIII A).

2.2.4.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix VIII B).

2.2.4.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix VIII C).

2.2.4.6 Test for B virus with rabbits

The pooled virus seed suspension shall be subject

to the test. If the test can not be conducted immediately, the samples shall be stored at or below -20°C . At least five healthy rabbits weighing 1.5-2.5 kg each are used for the test. Each rabbit receives 10 ml of sample, of which 9 ml is for subcutaneous injection, the remaining 1.0 ml is for intracutaneous injections at multiple sites. Observe the rabbits for 3 weeks. At the end of observation period, the number of survivals shall be not less than 80% of the test animals. The test is considered satisfactory if signs of B virus or other viral infections are found. Autopsy shall be carried out if any of the test rabbits dies 24 hours after inoculation or the animals manifest symptoms and signs suggestive of B virus infection. Keep the tissue specimens of nervous system and viscera of the dead rabbits for further examination, and emulsify the brain tissue into 10% suspension that is used to inject another five healthy rabbits with the same method mentioned above.

2.2.4.7 Test for immunogenicity

Inoculate at least thirty poliomyelitis-susceptible children (with the antibody titer $<1:4$, before immunization) as routine practice with the original vaccine prepared from the master seed virus. Determine the neutralization antibody titer of serum samples taken before and one month after inoculation. The seroconversion rate shall be not less than 95%.

2.2.4.8 Test for neurovirulence in monkeys

It complies with the neurovirulence test in monkeys (Appendix XI L).

2.2.4.9 rct-Marker test

The suspension of monovalent virus is titrated in cell cultures incubated at $36^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$, separately. The t-control (seed virus for production or vaccine known to be safe to susceptible human) shall be set up. The rct-Marker for the monovalent virus suspension is considered qualified if the titer difference resulted from the titrations which are incubated at $36^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ is not lower than 5.0 lg for both the monovalent virus suspension and the t-control.

2.2.4.10 Test for nucleotide sequence of SV40

Carry out the test for nucleotide sequence of SV40 (Appendix IX H). The result shall be negative.

2.2.5 Storage of virus seeds

Add magnesium chloride to the liquid virus seed to a final concentration of 1 mol/L. The liquid virus seeds shall be kept at or below -60°C .

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

MEM containing lacto-albumin hydrolysate and a quantity of inactivated calf serum or other suitable

media can be used for cell culture medium. The quality of calf serum shall comply with the requirements in Appendix XIII D. MEM or other suitable media without calf serum can be used as maintenance medium.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix XIII C).

2.3.4 Inoculation and incubation

Inoculate seed virus proportional to the cell density. After virus inoculation, incubate the cell cultures at $(33 \pm 0.5)^{\circ}\text{C}$. Normally the CPE shall develop fully between 40 and 96 hours after inoculation.

2.3.5 Virus harvest

After clarification through filtration, the virus suspensions are pooled to make the monovalent bulk.

2.3.6 Pooling or concentration

The monovalent virus bulks can be pooled or concentrated.

2.3.7 Control tests on monovalent bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Add magnesium chloride into the monovalent bulk to a final concentration of 1 mol/L to make the monovalent final bulk. The final bulks of individual three types are mixed in a certain proportion to make the trivalent final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Vaccine dragee preparation

Mix a quantity of trivalent vaccine bulk with excipient proportionally in the rolling pot. During the process of rolling the temperature in the workshop shall be at or below 18°C .

2.5.2 Defining batches

The Requirements for Defining Batches of Biologics shall apply. The dragees made on the same date can be defined as one lot. The dragees made in different pots shall be defined as different sub-lots.

2.5.3 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.4 Specifications

1 g per single dragee containing not less than 5.95 lg CCID₅₀ in total of live polioviruses, of which not less than 5.8 lg CCID₅₀ for type I, not less than 4.8 lg CCID₅₀ for type II and not less than 5.3 lg CCID₅₀ for type III.

2.5.5 Packaging

The Requirements for Packaging of Biologics shall

apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Identity test

See Section 2.2.4.1.

3.1.2 Virus titration

See Section 2.2.3.2. The titer shall be not less than 6.5 lg CCID₅₀/ml.

3.1.3 Test for neurovirulence in monkeys

It complies with the neurovirulence test in monkeys (Appendix XI L).

3.1.4 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.5 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control tests on final bulk

3.2.1 Virus titration

See Section 2.2.4.2. The titer of trivalent vaccine of final bulk shall be not less than 7.15 lg CCID₅₀/ml in total, of which not less than 7.0 lg CCID₅₀/ml for type I, not less than 6.0 lg CCID₅₀/ml for type II and not less than 6.5 lg CCID₅₀/ml for type III.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

200-300 dragees are sampled from each rolling pot.

3.3.1 Identity test

Mix the test sample with a quantity of the mixed antiserum of three types. The mixture is incubated at 35-37°C for 1-2 hours and then inoculated onto Hep-2 or other appropriate cell cultures. After incubation at 35-36°C for 7 days, no CPE shall be found. Both the results of controls of cell cultures and antiserum shall be negative. The control of virus shall be positive.

3.3.2 Inspection on final product

The vaccine is in a form of white solid dragee.

3.3.3 Weight variation

Each time twenty dragees shall be sampled and weighed on each dragee basis. The weight for trivalent vaccine is 1 g ± 0.15 g per dragee.

3.3.4 Virus titration

One hundred dragees from every three to four pots shall be combined into one batch for the test. Dissolve 100 dragees in 1000 ml of Earle solution to make 1 : 10 dilution. Suitable dilutions are employed for virus titration by CPE method.

For trivalent vaccine the total virus content shall be determined. At the same time the virus titer of each type shall be determined separately by means of neutralization. However, the cross-inhibition

of heterotypic antibody shall be precisely determined in advance in order to calibrate the result of virus titration. See Section 2.2.4.2. The total virus content in trivalent vaccine shall be not less than 5.95 lg CCID₅₀ per dragee, of which the live poliomyelitis virus contents for type I, type II and type III shall be not less than 5.8 lg CCID₅₀, not less than 4.8 lg CCID₅₀ and not less than 5.3 lg CCID₅₀, respectively.

3.3.5 Thermostability test

Before release the final product is subject to thermostability test, and the virus titration shall be carried out simultaneously. The vaccine that has been exposed at 37°C for 48 hours shall be titrated according to Section 2.2.4.2. The virus titer shall be not less than 5.0 lg CCID₅₀. The loss of titer of the exposed vaccine shall be not more than 1.0 lg.

3.3.6 Uniformity of virus distribution

At least ten dragees shall be sampled from each batch to validate the uniformity of virus distribution between dragees, which shall be tested on each dragee basis. The difference of virus content between dragees shall be not more than 0.5 lg.

3.3.7 Test for contaminating microorganisms

At least ten dragees shall be sampled from each rolling pot, and the samples of dragees prepared on the same date can be mixed for testing. The number of contaminating microorganisms in one dragee of trivalent vaccine shall not exceed 300.

3.3.8 Test for pathogenic bacteria

No β-hemolytic streptococcus or pathogenic intestinal bacteria, *E. coli* shall be present in the vaccine.

3.3.8.1 Test for β-hemolytic streptococcus

Inoculate 0.5 ml of 1 : 10 diluted vaccine into a test tube of broth. After incubation at 37°C for 24 hours, transfer the cultured broth onto a blood plate by streaks and incubate at 37°C for 24 hours. No β-hemolytic streptococcus growth shall occur (if the source and subsidiary materials used in the product have passed the test, it is not necessary to test the final product).

3.3.8.2 Test for pathogenic intestinal bacteria

Inoculate 1.0 ml of 1 : 10 diluted vaccine into a test tube of GN medium or broth enrichment medium. After incubation at 37°C for 6-24 hours, transfer the cultured material on an indicator plate by streaks and incubate at 37°C for 24 hours. If Gram-negative bacteria are found, further determination is necessary to identify whether they are pathogenic intestinal bacteria.

3.3.8.3 Test for *E. coli*

Inoculate 2 ml of the sample into each of three test tubes of Kessler medium or MacConkey broth medium. Incubate at 37°C for 48 hours, no acid or gas shall be produced. If it does, further tests are necessary to identify if they are *E. coli*.

4 Storage, shipping and validity period

From the date when the virus titration proved qualified, the validity period of the vaccine shall be 24 months if stored at or below -20°C ; it shall be 5 months if stored at $2-8^{\circ}\text{C}$. Only one kind of storage temperature and validity period shall be prescribed on the label. The vaccine shall be shipped under refrigeration.

5 Package inserts

Directions for Use of Poliomyelitis Vaccine in Dragee Candy (Human Diploid Cell), Live

[Drug name]

Adopted name: Poliomyelitis Vaccine in Dragee Candy (Human Diploid Cell), Live

[Constituents and characters]

The vaccine is made from attenuated poliovirus strains (Types I, II & III) grown separately in human diploid cells. After cultivation virus suspension is harvested to prepare the dragee-candy vaccine. The trivalent vaccine is a white solid dragee candy.

[Eligibles]

Mainly for children at or above 2 months of age.

[Function and use]

The product can induce immunity against poliovirus in recipients following immunization. It is used to prevent poliomyelitis.

[Specifications]

1 g per single dragee containing not less than $5.95 \lg \text{CCID}_{50}$ in total of live polioviruses, of which not less than $5.8 \lg \text{CCID}_{50}$ for type I, not less than $4.8 \lg \text{CCID}_{50}$ for type II and not less than $5.3 \lg \text{CCID}_{50}$ for type III.

[Administration and dosage]

The primary immunization should start at 2 months of age. Three doses shall be administered orally in the 1st year of age at intervals of 4-6 weeks. One booster shall be given in the 4th year of age. One dragee is one single human dose. The administration is also recommended for other age groups in case of need.

[Adverse reactions]

Normally there are no adverse reactions after oral administration. A few recipients might have fever, nausea, vomiting, and diarrhea or skin rashes. No particular treatment is necessary. Symptomatic treatment could be helpful if needed.

[Contraindications]

- (1) Subjects with fever, acute infectious diseases.
- (2) Those with immunodeficiency diseases, or those undergoing immunosuppressive therapy.
- (3) Women in pregnancy.

[Precautions]

- (1) The vaccine is for oral intake exclusively; do not use it for injection.
- (2) The vaccine is a live virus vaccine, it is recommended to take the dragee with warm (not

higher than 37°C) water, do not take it with hot water.

[Storage and shipping]

Store and ship at or below -20°C or $2-8^{\circ}\text{C}$, protected from light.

[Packaging]

[Validity period]

24 months if stored at or below -20°C ; 5 months at $2-8^{\circ}\text{C}$ (Only one kind of storage temperature and validity period can be prescribed on the label).

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:

Address:

Zip code:

Tel:

Fax:

Web site:

Poliomyelitis (Live) Vaccine (Monkey Kidney Cell), Oral

The poliomyelitis vaccine is a preparation of live attenuated poliovirus (types I, II, and III) grown in monkey kidney cell cultures. After cultivation, the virus suspension is harvested and prepared in a liquid form of either monovalent or trivalent vaccine. It is used to prevent poliomyelitis.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

The primary monkey kidney cells are used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

Rhesus monkeys shall be selected and quarantined for at least 6 weeks. Monkeys from which the kidneys are extracted for preparing the cell cultures must be healthy and have never been used previously for other experiments. The monkeys shall be free from tuberculosis, B virus infection and other acute infectious diseases. They shall also be demonstrated to be free from the circulating antibodies against foamy viruses. The animals that have serious purulent lesions, neoplasms, and obvious pathologic changes in liver or kidneys shall

not be used in the vaccine production.

2.1.2 Cell substrate preparation

Extract the kidneys from healthy monkeys meeting the requirements in Section 2.1.1. After trypsinization incubate the dispersed cells at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 6-9 days to form monolayers. The cell cultures prepared from kidneys of one single monkey are defined as one cell lot.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The virus strains used as the seeds for the vaccine production shall be types I, II and III attenuated strains, such as Sabin strain (types I, II and III), purified Sabin strain (types I, II and III), Zhong III₂ strain or other approved strains.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.2.2.1 Primary seed lot

Strains of Sabin types I, II, III and Zhong III₂ are prepared and are preserved by the original researchers.

2.2.2.2 Master seed lot

A batch of virus suspension of uniform composition, which is prepared by subculture of one to two passages from the primary seed virus in fetal monkey kidney cells or human diploid cells, is defined as the master seed lot.

The passage level of the Sabin master seed is SO+1. The passage level of Zhong III₂ master virus seed is Zhong III₂ 1. The master virus seed of type III Pfizer strain is RSO1.

2.2.2.3 Working seed lot

A batch of virus suspension of uniform composition, which is prepared from the master seed virus by subculture of a single passage in fetal monkey kidney cell cultures or human diploid cells, is defined as the working seed lot.

2.2.3 Virus passage

For the passages from the primary seed lot to the working seed lot, Sabin I, II and other purified strains as well as Zhong III₂ shall not exceed three passages; Sabin III and other purified strains including Pfizer strain shall not exceed two passages.

The cell cultures used for the preparation of virus seed lot for the production of vaccine shall be limited to fetal monkey kidney cells or human diploid cells.

2.2.4 Control tests on virus seed lots

For master and working seed lots, comprehensive control tests described below shall be conducted, unless otherwise specified.

2.2.4.1 Identity test

A quantity of the virus sample is mixed with an equal volume of monovalent antiserum against poliovirus type I, type II or type III corre-

spondingly. After incubation at $35-37^{\circ}\text{C}$ for 1-2 hours, the incubated mixture is inoculated onto the cell cultures of monkey kidney, Hep-2 or other susceptible cells. Read the results following incubation at $35-36^{\circ}\text{C}$ for 7 days after inoculation. The virus type shall be identified serologically without any suspicions. The corresponding serum control and cell control shall be set up in parallel and their results shall be negative. The result of virus control must be positive.

2.2.4.2 Virus titration

Micro-titration method is adopted. Samples shall be diluted 10-fold serially. Inoculate virus suspensions at different dilutions onto monkey kidney cells, Hep-2 or other susceptible cells. Incubate the inoculated cell cultures at $35-36^{\circ}\text{C}$ for 7 days. The titer shall be not less than $6.5 \lg \text{CCID}_{50}/\text{ml}$. During virus titration the virus reference shall be titrated in parallel.

2.2.4.3 Sterility test

It complies with the test for sterility (Appendix XIII A).

2.2.4.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XIII B).

2.2.4.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix XIII C).

2.2.4.6 Test for B virus with rabbits

The pooled virus seed suspension shall be subject to the test. If the test can not be conducted immediately, the samples shall be stored at or below -20°C . At least five healthy rabbits weighing 1.5-2.5 kg each are used for the test. Each rabbit receives 10 ml of sample, of which 9 ml is for subcutaneous injection, the remaining 1.0 ml is for intracutaneous injections at multiple sites. Observe the rabbits for 3 weeks. At the end of observation period, the number of survivals shall be not less than 80% of the test animals. The test is considered satisfactory if signs of B virus or other viral infections are found. Autopsy shall be carried out if any of the test rabbits dies 24 hours after inoculation or the animals manifest symptoms and signs suggestive of B virus infection. Keep the tissue specimens of nervous system and viscera of the dead rabbits for further examination, and emulsify the brain tissue into 10% suspension that is used to inject another five healthy rabbits with the same method mentioned above.

2.2.4.7 Test for immunogenicity

Inoculate at least thirty poliomyelitis-susceptible children (with the antibody titer $<1:4$, before immunization) as routine practice with the original vaccine prepared from the master seed virus. Determine the neutralization antibody titer of serum samples taken before and one month after inoculation. The seroconversion rate shall be not

less than 95%.

2.2.4.8 Test for neurovirulence in monkeys

It complies with the neurovirulence test in monkeys (Appendix XI L).

2.2.4.9 rct-Marker test

The suspension of monovalent virus is titrated in cell cultures incubated at $36^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$, separately. The t-control (seed virus for production or vaccine known to be safe to susceptible human) shall be set up. The rct-Marker for the monovalent virus suspension is considered qualified if the titer difference resulted from the titrations which are incubated at $36^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ is not lower than 5.0 lg for both the monovalent virus suspension and the t-control.

2.2.4.10 Test for nucleotide sequence of SV₄₀

Carry out the test for nucleotide sequence of SV₄₀ (Appendix IX H). The result shall be negative.

2.2.5 Storage of virus seeds

Add magnesium chloride to the liquid virus seed to a final concentration of 1 mol/L. The liquid virus seeds shall be kept at or below -60°C .

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Earle solution containing lacto-albumin hydrolysate and a quantity of inactivated calf serum or other suitable media shall be used as the culture medium. The quality of calf serum shall comply with the requirements in Appendix XIII D. Earle solution without lacto-albumin hydrolysate and calf serum or other suitable media can be used as maintenance medium.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix XIII C).

2.3.4 Inoculation and incubation

Inoculate seed virus proportional to the cell density. After virus inoculation, incubate the cell cultures at $33^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Normally the CPE shall develop fully between 40 and 96 hours after inoculation.

2.3.5 Virus harvest

After clarification through filtration, the virus suspensions are pooled to make the monovalent bulk.

2.3.6 Pooling or concentration

The monovalent virus bulks can be pooled or concentrated.

2.3.7 Control tests on monovalent bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Add magnesium chloride into the monovalent bulk

to a final concentration of 1 mol/L to make the monovalent final bulk. The final bulks of individual three types are mixed in a certain proportion to make the trivalent final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1.0 ml per container. Two drops (equivalent to 0.1 ml) per single human dose containing not less than 6.15 lg CCID₅₀ in total of live poliovirus, on which not less than 6.0 lg CCID₅₀ for type I, not less than 5.0 lg CCID₅₀ for type II and not less than 5.5 lg CCID₅₀ for type III.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Identity test

See Section 2.2.4.1.

3.1.2 Virus titration

See Section 2.2.4.2. The titer shall be not less than 6.5 lg CCID₅₀/ml.

3.1.3 Test for neurovirulence in monkeys

It complies with the neurovirulence test in monkeys (Appendix XI L).

3.1.4 Test for nucleotide sequence of SV40

Carry out the test for nucleotide sequencing (Appendix IX H). The result shall be negative.

3.1.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.6 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

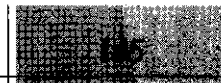
3.2 Control tests on final bulk

3.2.1 Virus titration

See Section 2.2.4.2. The titer of monovalent vaccine final bulk shall be not less than 6.5 lg CCID₅₀/ml. The titer of trivalent vaccine final bulk shall be not less than 7.15 lg CCID₅₀/ml in total, of which not less than 7.0 lg CCID₅₀/ml for type I, not less than 6.0 lg CCID₅₀/ml for type II and not less than 6.5 lg CCID₅₀/ml for type III.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).



3.3 Control tests on final product

3.3.1 Identity test

Mix the test sample with a quantity of the mixed antiserum of three types. The mixture is incubated at 35-37°C for 1-2 hours and then inoculated onto Hep-2 or other appropriate cell cultures. After incubation at 35-36°C for 7 days, no CPE shall be found. Both the results of controls of cell cultures and antiserum shall be negative. The result of control of virus shall be positive.

3.3.2 Inspection on final containers

The vaccine is a clear, reddish orange liquid free of foreign matters.

3.3.3 Virus titration

See Section 2.2.4.2. For trivalent vaccine, each single human dose (0.1 ml) contains not less than 6.15 lg CCID₅₀, of which not less than 6.0 lg CCID₅₀ for type I, not less than 5.0 lg CCID₅₀ for type II and not less than 5.5 lg CCID₅₀ for type III.

3.3.4 Thermostability test

Before release the final product is subject to thermostability test, and the virus titration shall be carried out simultaneously. The vaccine that has been exposed at 37°C for 48 hours shall be titrated according to Section 2.2.4.2. The loss of titer of a single human dose of the exposed vaccine shall be not more than 0.5 lg.

3.3.5 Sterility test

It complies with the test for sterility (Appendix X A).

4 Storage, shipping and validity period

From the date when the virus titrations proved qualified, the validity period of the vaccine shall be 24 months if stored at or below -20°C; shall be 12 months if stored at 2-8°C. Only one kind of storage temperature and validity period shall be prescribed on the label. The vaccine shall be shipped under refrigeration.

5 Package inserts

Directions for Use of Poliomyelitis (Live) Vaccine (Monkey Kidney Cell), Oral

[Drug name]

Adopted name: Poliomyelitis (Live) Vaccine (Monkey Kidney Cell), Oral

[Constituents and characters]

The vaccine is made from attenuated poliovirus strains (types I, II & III) grown separately in primary monkey kidney cell cultures. After cultivation, virus suspension is harvested to prepare the vaccine. The vaccine is a clear, reddish orange liquid.

[Eligibles]

Mainly for poliomyelitis-susceptible children at or above 2 months of age.

[Function and use]

The product can induce immunity against poliovirus in

recipients following immunization. It is used to prevent poliomyelitis.

[Specifications]

1.0 ml per container. Two drops (equivalent to 0.1 ml) per single human dose containing not less than 6.15 lg CCID₅₀ in total of live poliovirus, of which not less than 6.0 lg CCID₅₀ for type I, not less than 5.0 lg CCID₅₀ for type II and not less than 5.5 lg CCID₅₀ for type III.

[Administration and dosage]

The primary immunization should start at 2 months of age. Three doses shall be administered orally in the 1st year of age at intervals of 4-6 weeks. One booster shall be given in the 4th year of age. Two drops (equivalent to 0.1 ml) is one single human dose. The administration is also recommended for other age groups in case of need.

[Adverse reactions]

Normally there are no adverse reactions after oral administration. A few recipients might have fever, nausea, vomiting, and diarrhea or skin rashes. No particular treatment is necessary. Symptomatic treatment could be helpful if needed.

[Contraindications]

- (1) Subjects with fever, acute infectious diseases.
- (2) Those with immunodeficiency diseases, or those receiving immunosuppressive therapy.
- (3) Women in pregnancy.

[Precautions]

The vaccine is a live virus vaccine, it is recommended to take the dragee with warm (not higher than 37°C) water, do not take it with hot water.

[Storage and shipping]

Store and ship at or below -20°C or 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months if stored at or below -20°C; 12 months at 2-8°C (Only one kind of storage temperature and validity period can be prescribed on the label).

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:
Address:
Zip code:
Tel:
Fax:
Web site:

Poliomyelitis Vaccine in Dragee Candy (Monkey Kidney Cell), Live

The poliomyelitis vaccine in dragee candy is a preparation of live attenuated poliovirus (types I, II, and III) grown in primary monkey kidney cell cultures. After cultivation the virus suspension is harvested and made into a dragee candy form. It is used to prevent poliomyelitis.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Cell substrates for vaccine production

The primary monkey kidney cells are used for the vaccine production.

2.1.1 Management and control tests on cell substrates

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

Rhesus monkeys shall be selected and quarantined for at least 6 weeks. Monkeys from which the kidneys are extracted for preparing the cell cultures must be healthy and have never been used previously for other experiments. The monkeys shall be free from tuberculosis, B virus infection and other acute infectious diseases. They shall also be demonstrated to be free from the circulating antibodies against foamy viruses. The animals that have serious purulent lesions, neoplasms, and obvious pathologic changes in liver or kidneys shall not be used in the vaccine production.

2.1.2 Cell substrate preparation

Extract the kidneys from healthy monkeys meeting the requirements in Section 2.1.1. After trypsinization incubate the dispersed cells at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 6-9 days to form monolayers. The cell cultures prepared from kidneys of one single monkey are defined as one cell lot.

2.2 Virus seeds

2.2.1 Name and origin of virus strains

The virus strain used as the seeds for the vaccine production shall be Types I, II and III attenuated strains, such as Sabin strain (types I, II and III), purified Sabin strain (types I, II and III), Zhong III₂ strain or other approved strains.

2.2.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.2.2.1 Primary seed lot

Strains of Sabin types I, II, III and Zhong III₂ are prepared and are preserved by the original researchers.

2.2.2.2 Master seed lot

A batch of virus suspension of uniform composition, which is prepared by subculture for one to two passages from the primary seed virus in fetal monkey kidney cells or human diploid cells, is defined as the master seed lot.

The passage level of the Sabin master seed is SO+1. The passage level of Zhong III₂ master seed is Zhong III₂ 1. The master virus seed of type III Pfizer strain is RSO1.

2.2.2.3 Working seed lot

A batch of virus suspension of uniform composition, which is prepared from the master seed virus by subculture for a single passage in fetal monkey kidney cell cultures or human diploid cells, is defined as the working seed lot.

2.2.3 Virus passage

For the passages from the primary seed lot to the working seed lot, Sabin I, II and other purified strains as well as Zhong III₂ shall not exceed three passages; Sabin III and other purified strains including Pfizer strain shall not exceed two passages.

The cell cultures used for the preparation of virus seed lot for the production of vaccine shall be limited to fetal monkey kidney cells or human diploid cells.

2.2.4 Control tests on virus seed lots

For master and working seed lots, comprehensive control tests described below shall be conducted, unless otherwise specified.

2.2.4.1 Identity test

A quantity of the virus sample is mixed with an equal volume of monovalent antiserum against poliovirus type I, type II or type III correspondingly. After incubation at $35-37^{\circ}\text{C}$ for 1-2 hours, the incubated mixture is inoculated onto the cell cultures of monkey kidney, Hep-2 or other susceptible cells. Read the results following incubation at $35-36^{\circ}\text{C}$ for 7 days after inoculation. The virus type shall be identified serologically without any suspicions. The corresponding serum control and cell control shall be set up in parallel and their results shall be negative. The result of virus control must be positive.

2.2.4.2 Virus titration

Micro-titration method is adopted. Samples shall be diluted 10-fold serially. Inoculate virus suspensions at different dilutions onto monkey kidney cells, Hep-2 or other susceptible cells. Incubate the inoculated cell cultures at $35-36^{\circ}\text{C}$ for 7 days. The titer shall be not less than $6.5 \lg \text{CCID}_{50}/\text{ml}$. During virus titration the virus reference shall be titrated in parallel.

2.2.4.3 Sterility test

It complies with the test for sterility (Appendix

Ⅺ A).

2.2.4.4 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix Ⅺ B).

2.2.4.5 Tests for adventitious viruses

It complies with the tests for adventitious viruses (Appendix Ⅺ C).

2.2.4.6 Test for B virus with rabbits

The pooled virus seed suspension shall be subject to the test. If the test can not be conducted immediately, the samples shall be stored at or below -20°C . At least five healthy rabbits weighing 1.5-2.5 kg each are used for the test. Each rabbit receives 10 ml of sample, of which 9 ml is for subcutaneous injection, the remaining 1.0 ml is for intracutaneous injections at multiple sites. Observe the rabbits for 3 weeks. At the end of observation period, the number of survivals shall be not less than 80% of the test animals. The test is considered satisfactory if signs of B virus or other viral infections are found. Autopsy shall be carried out if any of the test rabbits dies 24 hours after inoculation or the animals manifest symptoms and signs suggestive of B virus infection. Keep the tissue specimens of nervous system and viscera of the dead rabbits for further examination, and emulsify the brain tissue into 10% suspension that is used to inject another five healthy rabbits with the same method mentioned above.

2.2.4.7 Test for immunogenicity

Inoculate at least thirty poliomyelitis-susceptible children (with the antibody titer $<1:4$, before immunization) as routine practice with the original vaccine prepared from the master seed virus. Determine the neutralization antibody titer of serum samples taken before and one month after inoculation. The seroconversion rate shall be not less than 95%.

2.2.4.8 Test for neurovirulence in monkeys

It complies with the neurovirulence test in monkeys (Appendix Ⅺ L).

2.2.4.9 rct-Marker test

The suspension of monovalent virus is titrated in cell cultures incubated at $36^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$, separately. The t-control (seed virus for production or vaccine known to be safe to susceptible human) shall be set up. The rct-Marker for the monovalent virus suspension is considered qualified if the titer difference resulted from the titrations which are incubated at $36^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ is not lower than 5.0 lg for both the monovalent virus suspension and the t-control.

2.2.4.10 Test for nucleotide sequence of SV40

Carry out the test for nucleotide sequence of SV40 (Appendix Ⅺ H). The result shall be negative.

2.2.5 Storage of virus seeds

Add magnesium chloride to the liquid virus seed to

a final concentration of 1 mol/L. The liquid virus seeds shall be kept at or below -60°C .

2.3 Bulk

2.3.1 Cell substrate preparation

See Section 2.1.2.

2.3.2 Culture medium

Earle solution containing lacto-albumin hydrolysate and a quantity of inactivated calf serum or other suitable media shall be used as the culture medium. The quality of calf serum shall comply with the requirements in Appendix Ⅺ D. Earle solution without lacto-albumin hydrolysate and calf serum or other suitable media can be used as maintenance medium.

2.3.3 Tests for adventitious viruses in control cells

It complies with the tests for adventitious viruses (Appendix Ⅺ C).

2.3.4 Inoculation and incubation

Inoculate seed virus proportional to the cell density. After virus inoculation, incubate the cell cultures at $33^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Normally the CPE shall develop fully between 40 and 96 hours after inoculation.

2.3.5 Virus harvest

After clarification through filtration, the virus suspensions are pooled to make the monovalent bulk.

2.3.6 Pooling or concentration

The monovalent virus bulks can be pooled or concentrated.

2.3.7 Control tests on monovalent bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Add magnesium chloride into the monovalent bulk to a final concentration of 1 mol/L to make the monovalent final bulk. The final bulks of individual three types are mixed in a certain proportion to make the trivalent final bulk.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Vaccine dragee preparation

Mix a quantity of trivalent vaccine bulk with excipient proportionally in the rolling pot. During the process of rolling the temperature in the workshop shall be at or below 18°C .

2.5.2 Defining batches

The Requirements for Defining Batches of Biologics shall apply. The dragees made on the same date can be defined as one lot. The dragees made in different pots shall be defined as different sub-lots.

2.5.3 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.4 Specifications

1 g per single dragee containing not less than 5.95 lg CCID₅₀ in total of live poliovirus, of which not less than 5.8 lg CCID₅₀ for type I, not less than 4.8 lg CCID₅₀ for type II and not less than 5.3 lg CCID₅₀ for type III.

2.5.5 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Identity test

See Section 2.2.4.1.

3.1.2 Virus titration

See Section 2.2.4.2. The titer shall be not less than 6.5 lg CCID₅₀/ml.

3.1.3 Test for neurovirulence in monkeys

It complies with the neurovirulence test in monkeys (Appendix XI L).

3.1.4 Test for nucleotide sequence of SV40

Carry out the test for nucleotide sequencing (Appendix IX H). The result shall be negative.

3.1.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.6 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix XII B).

3.2 Control tests on final bulk

3.2.1 Virus titration

See Section 2.2.4.2. The titer of monovalent vaccine final bulk shall be not less than 6.5 lg CCID₅₀/ml. The titer of trivalent vaccine final bulk shall be not less than 7.15 lg CCID₅₀/ml in total, of which not less than 7.0 lg CCID₅₀/ml for type I, not less than 6.0 lg CCID₅₀/ml for type II and not less than 6.5 lg CCID₅₀/ml for type III.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

200-300 dragees are sampled from each rolling pot.

3.3.1 Identity test

Mix the test sample with a quantity of the mixed antiserum of three types. The mixture is incubated at 35-37°C for 1-2 hours and then inoculated onto Hep-2 or other appropriate cell cultures. After incubation at 35-36°C for 7 days, no CPE shall be found. Both the results of controls of cell cultures and antiserum shall be negative. The result of control of virus shall be positive.

3.3.2 Inspection on final product

The vaccine is in a form of white solid dragee.

3.3.3 Weight variation

Each time twenty dragees shall be sampled and

weighed on each dragee basis. The weight for trivalent vaccine is 1 g ± 0.15 g per dragee.

3.3.4 Virus titration

One hundred dragees from every three to four pots shall be combined into one batch for the test. Dissolve 100 dragees in 1000 ml of Earle solution to make 1 : 10 dilution. Suitable dilutions are employed for virus titration by CPE method.

For trivalent vaccine, the total virus content shall be determined. At the same time the virus titer of each type shall be determined separately by means of neutralization. However, the cross-inhibition of heterotypic antibody shall be precisely determined in advance in order to calibrate the result of virus titration. See Section 2.2.4.2. The total virus content in trivalent vaccine shall be not less than 5.95 lg CCID₅₀ per dragee, of which the live poliomyelitis virus content for type I, type II and type III shall be not less than 5.8 lg CCID₅₀, not less than 4.8 lg CCID₅₀ and not less than 5.3 lg CCID₅₀, respectively.

3.3.5 Thermostability test

Before release the final product is subject to thermostability test, and the virus titration shall be carried out simultaneously. The vaccine that has been exposed at 37°C for 48 hours shall be titrated according to Section 2.2.4.2. The virus titer shall be not less than 5.0 lg CCID₅₀. The loss of titer of the exposed vaccine shall be not more than 1.0 lg.

3.3.6 Uniformity of virus distribution

Samples of ten dragees at least shall be taken to validate the uniformity of virus distribution between dragees, which shall be tested on each dragee basis. The difference of virus content between dragees shall be not more than 0.5 lg.

3.3.7 Test for contaminating microorganisms

At least ten dragees shall be sampled from each rolling pot, and the samples of dragees prepared on the same date can be mixed for testing. The number of contaminating microorganisms in one dragee of trivalent vaccine shall not exceed 300.

3.3.8 Test for pathogenic bacteria

No β-hemolytic streptococcus or pathogenic intestinal bacteria, *E. coli* shall be present in the vaccine.

3.3.8.1 Test for β-hemolytic streptococcus

Inoculate 0.5 ml of 1 : 10 diluted vaccine into a test tube of broth. After incubation at 37°C for 24 hours, transfer the cultured broth onto a blood plate by streaks and incubate at 37 °C for 24 hours. No β-hemolytic streptococcus growth shall occur (if the source and subsidiary materials used in the product have passed the test, it is not necessary to test the final product).

3.3.8.2 Test for pathogenic intestinal bacteria

Inoculate 1.0 ml of 1 : 10 diluted vaccine into a test tube of GN medium or broth enrichment

medium. After incubation at 37°C for 6-24 hours, transfer the cultured material on an indicator plate by streaks and incubate at 37°C for 24 hours. If Gram-negative bacteria are found, further determination is necessary to identify whether they are pathogenic intestinal bacteria.

3.3.8.3 Test for *E. coli*

Inoculate 2 ml of the sample into each of three test tubes of Kessler medium or MacConkey broth medium. Incubate at 37°C for 48 hours, no acid or gas shall be produced. If it does, further tests are necessary to identify if they are *E. coli*.

4 Storage, shipping and validity period

From the date when the virus titration proved qualified, the validity period of the vaccine shall be 24 months if stored at or below -20°C; it shall be 5 months if stored at 2-8°C. Only one kind of storage temperature and validity period shall be prescribed on the label. The vaccine shall be shipped under refrigeration.

5 Package inserts

Directions for Use of Poliomyelitis Vaccine in Dragee Candy (Monkey Kidney Cell), Live

[Drug name]

Adopted name: Poliomyelitis Vaccine in Dragee Candy (Monkey Kidney Cell), Live

[Constituents and characters]

The vaccine is made from attenuated poliovirus strains (types I, II & III) grown separately in primary monkey kidney cell cultures. After cultivation, virus suspension is harvested to prepare the dragee-candy vaccine. The trivalent vaccine is a white solid dragee candy.

[Eligibles]

Mainly for children at or above 2 months of age.

[Function and use]

The product can induce immunity against poliovirus in recipients following immunization. It is used to prevent poliomyelitis.

[Specifications]

1 g per single dragee containing not less than 5.95 lg CCID₅₀ in total of live poliovirus, of which not less than 5.8 lg CCID₅₀ for type I, not less

than 4.8 lg CCID₅₀ for type II and not less than 5.3 lg CCID₅₀ for type III.

[Administration and dosage]

The primary immunization should start at 2 months of age. Three doses shall be administered orally in the 1st year of age at intervals of 4-6 weeks. One booster shall be given in the 4th year of age. One dragee is one single human dose. The administration is also recommended for other age groups in case of need.

[Adverse reactions]

Normally there are no adverse reactions after oral administration. A few recipients might have fever, nausea, vomiting, and diarrhea or skin rashes. No particular treatment is necessary. Symptomatic treatment could be helpful if needed.

[Contraindications]

- (1) Subjects with fever, acute infectious diseases.
- (2) Those with immunodeficiency diseases, or those receiving immunosuppressive therapy.
- (3) Women in pregnancy.

[Precautions]

The vaccine is a live virus vaccine, it is recommended to take the dragee with warm (not higher than 37°C) water, do not take it with hot water.

[Storage and shipping]

Store and ship at or below -20°C or 2-8°C, protected from light.

[Packaging]

[Validity period]

24 months if stored at or below -20°C; 5 months at 2-8°C (only one kind of storage temperature and validity period can be prescribed on the label).

[Standard for implementation]

[Product license number]

[Manufacturer]

Name:
Address:
Zip code:
Tel:
Fax:
Web site:

II Biologics for Therapeutic Use

Diphtheria Antitoxin

Diphtheria antitoxin is a liquid preparation containing antitoxic globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with diphtheria toxoid. The preparation is used for prevention and treatment of diphtheria.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply. Blood shall be collected when the potency of immunoserum is not less than 1100 IU/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XII A).

2.3 Bulk

2.3.1 Source plasma

The diphtheria antitoxin potency of source plasma shall be not less than 1000 IU/ml (Appendix XII E). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative. The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile for water injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1000 IU per container (for prophylactic use); 8000 IU per container (for therapeutic use).

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall

be not more than 4 µg/ml (Appendix IX I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI E).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI E, and the result shall demonstrate that the test sample can neutralize diphtheria toxin in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with diphtheria toxoid.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless or light yellow liquid, free from foreign matters. After storage for a long time, a trace of precipitate may occur, which can be dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.5 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2%, and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 50% in antitoxin for prophylactic use, and not less than 60% in antitoxin for therapeutic use (Appendix VIII F).

3.3.5 Antibody potency

The potency of diphtheria antitoxin shall be not less than 2000 IU/ml for prophylactic use, and not less than 3000 IU/ml for therapeutic use. The specific activity shall be not less than 30000 IU/g of protein for prophylactic use, and not less than 40000 IU/g of protein for therapeutic use (Appendix XI E). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Diphtheria Antitoxin, Freeze-dried

Freeze-dried diphtheria antitoxin is a preparation containing antitoxic globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with diphtheria toxoid. The preparation is used for prevention and treatment of diphtheria.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for

production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum is not less than 1100 IU/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XIII A).

2.3 Bulk

2.3.1 Source plasma

The diphtheria antitoxin potency of source plasma shall be not less than 1000 IU/ml (Appendix XI E). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative. The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content

according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

1000 IU per container (for prophylactic use);
8000 IU per container (for therapeutic use).

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI E).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

Other than the determination of residual moisture, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI E, and the result shall demonstrate that the test sample can neutralize diphtheria toxin in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with diphtheria toxoid.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparation looks like a white or light yellow crisp cake. It shall be completely reconstituted with the stated amount of water for injection within 15 minutes by shaking gently. After reconstitution, it shall be a clear, colourless or light yellow liquid, free from foreign matters.

3.3.3 Chemical tests**3.3.3.1 Moisture content**

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity**3.3.4.1 Albumin detection**

Dilute sample to a protein content of 2%, and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 50% in antitoxin for prophylactic use, and not less than 60% in antitoxin for therapeutic use (Appendix VIII F).

3.3.5 Antibody potency

The potency of diphtheria antitoxin shall be not less than 2000 IU/ml for prophylactic use, and not less than 3000 IU/ml for therapeutic use. The specific activity shall be not less than 30000 IU/g of protein for prophylactic use, and not less than 40000 IU/g of protein for therapeutic use (Appendix XI E). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Diluent

The diluent for reconstitution of final product is sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light.

The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Tetanus Antitoxin

Tetanus antitoxin is a liquid preparation containing antitoxic globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with tetanus toxoid. The preparation is used for prevention and treatment of tetanus.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing**2.1 Antigen and adjuvant**

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply.

2.2 Animals used for immunization and plasma**2.2.1 Animals used for immunization**

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply. Blood shall be collected when the potency of immunoserum is not less than

1100 IU/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XIII A).

2.3 Bulk

2.3.1 Source plasma

The tetanus antitoxin potency of source plasma shall be not less than 1000 IU/ml (Appendix XII F). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

1500 IU per container (for prophylactic use);
10000 IU per container (for therapeutic use).

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance
The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI F).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI F, and the result shall demonstrate that the test sample can neutralize tetanus toxin in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with tetanus toxoid.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless or light yellow liquid, free from foreign matters. After storage for a long time, a trace of precipitate may occur, which can be dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.5 Preservative content

If any of the following preservatives is added, it shall be:
not more than 0.1 g/L for the content of thimerosal

(Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute the test sample to a protein content of 2% and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 50% in antitoxin for prophylactic use, and not less than 60% in antitoxin for therapeutic use (Appendix VIII F).

3.3.5 Antibody potency

The potency of tetanus antitoxin shall be not less than 2000 IU/ml for prophylactic use, and not less than 3000 IU/ml for therapeutic use. The specific activity shall be not less than 35000 IU/g of protein for prophylactic use, and not less than 45000 IU/g of protein for therapeutic use (Appendix XI F). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Tetanus Antitoxin, Freeze-dried

Freeze-dried tetanus antitoxin is a preparation containing antitoxic globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with tetanus toxoid. The preparation is used for prevention and treatment of tetanus.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum is not less than 1100 IU/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XIII A).

2.3 Bulk

2.3.1 Source plasma

The tetanus antitoxin potency of source plasma shall be not less than 1000 IU/ml (Appendix XI F). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

1500 IU per container (for prophylactic use);
10000 IU per container (for therapeutic use).

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI F).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of residual moisture, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI F, and the result shall demonstrate that the test sample can neutralize tetanus toxin in animals. Alternatively, carry out the double

immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with tetanus toxoid.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparation looks like a white or light yellow crisp cake. It shall be completely reconstituted with the stated amount of water for injection within 15 minutes by shaking gently. After reconstitution, it shall be a clear, colourless or light yellow liquid, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VIII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VIII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VIII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute the test sample to a protein content of 2% and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 50% in antitoxin for prophylactic use, and not less than 60% in antitoxin for therapeutic use (Appendix VII F).

3.3.5 Antibody potency

The potency of tetanus antitoxin shall be not less than 2000 IU/ml for prophylactic use, and not less than 3000 IU/ml for therapeutic use. The specific activity shall be not less than 35000 IU/g of protein for prophylactic use and not less than 45000 IU/g of protein for therapeutic use

(Appendix XI F). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance
The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Diluent

The diluent for reconstitution of final product is sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Gas-gangrene Antitoxin (Mixed)

Mixed gas-gangrene antitoxin is a liquid preparation containing polyvalent antitoxic globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized separately with the toxin or toxoid derived from *Cl. perfringens*, *Cl. oedematiens* (*novyi*), *Cl. septicum* and *Cl. histolyticum*. The preparation is used for prevention and treatment of gas-gangrene.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply. Blood shall be collected when the potency of immunoserum meets the following requirements:

300 IU/ml or more for *Cl. perfringens*;

700 IU/ml or more for *Cl. oedematiens*;

350 IU/ml or more for *Cl. Septicum*;

700 IU/ml or more for *Cl. histolyticum*.

A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XII A).

2.3 Bulk

2.3.1 Source plasma

The gas-gangrene antitoxin potency of source plasma (Appendix XI G) shall meet the following requirements:

250 IU/ml or more for *Cl. perfringens*;

300 IU/ml or more for *Cl. septicum*;

550 IU/ml or more for *Cl. Oedematiens*;

550 IU/ml or more for *Cl. histolyticum*.

If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration. Three bulk antitoxins shall be mixed in a proportion of international units as follows: *Cl. perfringens* : *Cl. oedematiens* : *Cl. septicum* = 2 : 2 : 1. One

portion of *Cl. histolyticum* antitoxin may be added to the above mixture if necessary.

2.4.2 Control tests on final bulk
See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

5000 IU of mixed gas-gangrene antitoxin per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI G).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI G, and the result shall demonstrate that the test sample can neutralize relevant gas-gangrene toxins in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with relevant gas-gangrene toxins or toxoids, separately.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The product shall be a clear, colourless or light yellow liquid, free from foreign matters. After storage for a long time, a trace of precipitate may occur, which can be dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.5 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VIII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix VI B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

The potency of gas-gangrene antitoxin shall be not less than 1000 IU/ml (Appendix XI G). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the

date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Gas-gangrene Antitoxin (Mixed), Freeze-dried

Mixed gas-gangrene antitoxin is a freeze-dried preparation containing polyvalent antitoxic globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized separately with the toxin or toxoid derived from *Cl. perfringens*, *Cl. oedematiens* (*novyi*), *Cl. septicum* and *Cl. histolyticum*. The preparation is used for prevention and treatment of gas-gangrene.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply. Blood shall be collected when the potency of immunoserum meets the following requirements:

300 IU/ml or more for *Cl. perfringens*;
700 IU/ml or more for *Cl. oedematiens*;
350 IU/ml or more for *Cl. septicum*;
700 IU/ml or more for *Cl. histolyticum*.

A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix III A).

2.3 Bulk

2.3.1 Source plasma

The gas-gangrene antitoxin potency of source plasma (Appendix XI G) shall meet the following requirements:

250 IU/ml or more for *Cl. perfringens*;
300 IU/ml or more for *Cl. septicum*;
550 IU/ml or more for *Cl. oedematiens*;
550 IU/ml or more for *Cl. histolyticum*.

If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative. The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration. Three bulk antitoxins shall be mixed in a proportion of international units as follows: *Cl. perfringens* : *Cl. oedematiens* : *Cl. septicum* = 2 : 2 : 1. One portion of *Cl. histolyticum* antitoxin may be added to the above mixture if necessary.

2.4.2 Control tests on final bulk

See Section 3.2

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

5000 IU of mixed gas-gangrene antitoxin per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI G).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of residual moisture, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI G, and the result shall demonstrate that the test sample can neutralize relevant gas-gangrene toxins in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with relevant gas-gangrene toxins or toxoids, separately.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparation looks like a white or light yellow crisp cake. It shall be completely reconstituted with the stated amount of water for injection within 15 minutes by shaking gently. After reconstitution, it shall be a clear, colourless or light yellow liquid, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VIII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

The potency of gas-gangrene antitoxin shall be not less than 1000 IU/ml (Appendix XI G). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Diluent

The diluent for reconstitution of final product is sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Botulinum Antitoxins

Botulinum antitoxins, including six types of A,

B, C, D, E and F, are liquid preparations containing antitoxic globulins. Each type of antitoxin is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with the toxin or toxoid derived from the corresponding type of *Cl. botulinum*. The preparations are used for prevention and treatment of the corresponding type of botulism.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunoserum shall apply. Blood shall be collected when the potency of immunoserum meets the following requirements:

- 1500 IU/ml or more for type A;
- 800 IU/ml or more for type B;
- 300 IU/ml or more for type C;
- 800 IU/ml or more for type D;
- 800 IU/ml or more for type E;
- 300 IU/ml or more for type F.

A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XII A).

2.3 Bulk

2.3.1 Source plasma

The botulinum antitoxin potency of source plasma (Appendix XI H) shall meet the following requirements:

- 1000 IU/ml or more for type A;
- 600 IU/ml or more for type B;
- 200 IU/ml or more for type C;
- 600 IU/ml or more for type D;
- 600 IU/ml or more for type E;
- 200 IU/ml or more for type F.

If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

10000 IU per container for type A; 5000 IU per container for types B, C, D, E and F, respectively.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI H).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix

III D). The injecting dose of the preparations shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix III A).

3.3 Control tests on final product

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI H, and the result shall demonstrate that the test sample can neutralize the corresponding type of botulinum toxin or toxoid in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with the corresponding type of botulinum toxin or toxoid.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparations shall be clear, colourless or light yellow liquids, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.5 Preservative content

If any of the following preservatives is added, it shall be;

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix VI B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of

albumin.

3.3.4.2 F(ab)₂ content

The F(ab)₂ content shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

Carry out the test for potency (Appendix XI H). The potency and specific activity of each type of botulinum antitoxin shall be not less than the following standards;

Type	Potency	Specific activity
A	2000 IU/ml	20000 IU/g Pr
B	2000 IU/ml	20000 IU/g Pr
C	500 IU/ml	5000 IU/g Pr
D	2000 IU/ml	20000 IU/g Pr
E	1000 IU/ml	10000 IU/g Pr
F	500 IU/ml	5000 IU/g Pr

The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.3.7 Sterility test

It complies with the test for sterility (Appendix III A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix III D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix III F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Botulinum Antitoxins, Freeze-dried

Botulinum antitoxins, including six types of A, B, C, D, E and F, are freeze-dried preparations containing antitoxic globulins. Each type of antitoxin is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with the toxin or toxoid derived from the corresponding type of *Cl. botulinum*. The preparations are used for prevention and treatment of the corresponding type

of botulism.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum meets the following requirements:

- 1500 IU/ml or more for type A;
- 800 IU/ml or more for type B;
- 300 IU/ml or more for type C;
- 800 IU/ml or more for type D;
- 800 IU/ml or more for type E;
- 300 IU/ml or more for type F.

A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XII A).

2.3 Bulk

2.3.1 Source plasma

The botulinum antitoxin potency of source plasma (Appendix XI H) shall meet the following requirements:

- 1000 IU/ml or more for type A;
- 600 IU/ml or more for type B;
- 200 IU/ml or more for type C;
- 600 IU/ml or more for type D;
- 600 IU/ml or more for type E;
- 200 IU/ml or more for type F.

If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antitoxin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The temperature of the preparations during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

10000 IU per container for type A; 5000 IU per container for types B, C, D, E and F, respectively.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.1.2 Potency test

Carry out the test for potency (Appendix XI H).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparations shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix

XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted preparations shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI H, and the result shall demonstrate that the test sample can neutralize the corresponding type of botulinum toxin or toxoid respectively in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with the corresponding type of botulinum toxin or toxoid, separately.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparations look like white or light yellow crisp cake. They shall be completely reconstituted with the stated amount of water for injection within 15 minutes by shaking gently. After reconstitution, they shall be clear, colourless or light yellow liquids, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);
not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);
not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

The F(ab)₂ content shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

Carry out the test for potency (Appendix XI H). The potency and specific activity of each type of botulinum antitoxin shall be not less than the following standards:

Type	Potency	Specific activity
A	2000 IU/ml	20000 IU/g Pr
B	2000 IU/ml	20000 IU/g Pr
C	500 IU/ml	5000 IU/g Pr
D	2000 IU/ml	20000 IU/g Pr
E	1000 IU/ml	10000 IU/g Pr
F	500 IU/ml	5000 IU/g Pr

The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Diluent

The diluent for reconstitution of final product is sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Agkistrodon halys Antivenin, Equine

Agkistrodon halys antivenin is a freeze-dried

preparation containing antivenin globulins. The preparation is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with venoms or detoxified venoms of *Agkistrodon halys*. The preparation is used for treatment of victims bitten by *Agkistrodon halys*.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum reaches 180 U/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XIII A).

2.3 Bulk

2.3.1 Source plasma

The potency of source plasma shall be not less than 150 U/ml (Appendix IX I). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative. The purified bulk antivenin shall be stored at 2-8°C

and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3. 1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3. 2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

6000 U of *Agkistrodon halys* antivenin per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix IX I).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

Other than the determination of residual moisture, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix IX I, and the result shall demonstrate that the test sample can neutralize *Agkistrodon halys* venom in animals. Alternatively, carry out the double immunodiffusion test (Appendix VII C), and specific precipitation lines shall be produced with *Agkistrodon halys* venom.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VII C) by using the IgG of rabbit anti-horse plasma. The result shall demonstrate that the protein component is derived from horse serum.

3.3.2 Inspection on final containers

The preparation looks like a white or light yellow crisp cake. It shall be completely reconstituted with the stated amount of water for injection within 15 minutes on shaking gently. After reconstitution, it shall be a clear, colourless or light yellow liquid, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of metacresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix VI B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 60% (Appendix VII F).

3.3.5 Antibody potency

The potency of *Agkistrodon halys* antivenin shall be not less than 500 U/ml (Appendix XI I). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Diluent

The diluent for reconstitution of final product is sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Agkistrodon acutus Antivenin, Equine

Agkistrodon acutus antivenin is a freeze-dried preparation containing antivenin globulins. The preparation is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with venoms or detoxified venoms of *Agkistrodon acutus*. The preparation is used for treatment of victims bitten by *Agkistrodon acutus*.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immuno-sera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply

with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunosera reaches 60 U/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XII A).

2.3 Bulk

2.3.1 Source plasma

The potency of source plasma shall be not less than 50 U/ml (Appendix XI I). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antivenin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

2000 U of *Agkistrodon acutus* antivenin per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix XI I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI I).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of residual moisture, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI I, and the result shall demonstrate that the test sample can neutralize *Agkistrodon acutus* venom in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with *Agkistrodon acutus* venom.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C) by using the IgG of rabbit anti-horse plasma. The result shall demonstrate that the protein component is derived from horse serum.

3.3.2 Inspection on final containers

The preparation looks like a white or light yellow crisp cake. It shall be completely reconstituted with the stated amount of water for injection within 15 minutes on shaking gently. After

reconstitution, it shall be a clear, colourless or light yellow liquid, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of metacresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix VI B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

The potency of *Agkistrodon acutus* antivenin shall be not less than 180 U/ml (Appendix XI D).

The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

3.3.10 Diluent

The diluent for reconstitution of final product is

sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Bungarus multicinctus Antivenin, Equine

Bungarus multicinctus antivenin is a freeze-dried preparation containing antivenin globulins. The preparation is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with venoms or detoxified venoms of *Bungarus multicinctus*. The preparation is used for treatment of victims bitten by *Bungarus multicinctus*.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum reaches 300 U/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XIII A).

2.3 Bulk

2.3.1 Source plasma

The potency of source plasma shall be not less than 200 U/ml (Appendix XI D). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation



2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antivenin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

10000 U of *Bungarus multicinctus* antivenin per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI D).

3.1.3 Sterility test

It complies with the test for sterility (Appendix

XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of residual moisture, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix XI I, and the result shall demonstrate that the test sample can neutralize *Bungarus multicinctus* venom in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with *Bungarus multicinctus* venom.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C) by using the IgG of rabbit anti-horse plasma. The result shall demonstrate that the protein component is derived from horse serum.

3.3.2 Inspection on final containers

The preparation looks like a white or light yellow crisp cake. It shall be completely reconstituted with the stated amount of water for injection within 15 minutes on shaking gently. After reconstitution, it shall be a clear, colourless or light yellow liquid, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it

shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of metacresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix VI B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

The potency of *Bungarus multicinctus* antivenin shall be not less than 800 U/ml (Appendix XI D). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

3.3.10 Diluent

The diluent for reconstitution of final product is sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Naja naja (atra) Antivenin, Equine

Naja naja (atra) antivenin is a freeze-dried preparation containing antivenin globulins. The preparation is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with venoms or detoxified venoms of *Naja naja (atra)*. The preparation is used for treatment of victims bitten by *Naja naja*

(*atra*).

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum reaches 15 IU/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XIII A).

2.3 Bulk

2.3.1 Source plasma

The potency of source plasma shall be not less than 12 IU/ml (Appendix IX I). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antivenin shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final product, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.5.3 Specifications

1000 IU of *Naja naja (atra)* antivenin per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI D).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of residual moisture, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for identity as specified in Appendix IX I, and the result shall demonstrate

that the test sample can neutralize *Naja naja (atra)* venom in animals. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with *Naja Naja (atra)* venom.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C) by using the IgG of rabbit anti-horse plasma. The result shall demonstrate that the protein component is derived from horse serum.

3.3.2 Inspection on final containers

The preparation looks like a white or light yellow crisp cake. It shall be completely reconstituted with the stated amount of water for injection within 15 minutes on shaking gently. After reconstitution, it shall be a clear, colourless or light yellow liquid, free from foreign matters.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.4 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.6 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix VI B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

The potency of *Naja naja (atra)* antivenin shall be not less than 100 IU/ml (Appendix IX I). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX D).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Diluent

The diluent for reconstitution of final product is sterile water for injection.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 60 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Anthrax Antiserum

Anthrax antiserum is a liquid preparation containing anti-anthrax globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with the antigen of *Bacillus anthracis*. The preparation is used for prevention and treatment of anthrax.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum meets the

requirements. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XII A).

2.3 Bulk

2.3.1 Source plasma

The potency of source plasma shall comply with the requirements. If apparent hemolysis, bacterial contamination or other abnormalities occurs in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative. The purified bulk antiserum shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final products, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

20 ml per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance
The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.1.2 Potency test

Inject s.c. 0.5 ml of antiserum into each of eight guinea pigs weighing 350-400 g. Challenge each animal with 1 MLD of spores suspension of *Bacillus anthracis* (PNo. 2 strain) 24 hours after injection. As a control, four guinea pigs with the same body weight, which have not been injected with antiserum, shall be challenged each with 1 MLD. The test shall be judged as qualified if more than six of eight (75%) animals in the test group survive, and at least three of four animals in the control group die during the observation period of 14 days. It is allowable that the animal left in the control group dies or shows sign of illness later.

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals or specific precipitation

Perform the test for potency given in Section 3.1.2. Alternatively, carry out the double immunodiffusion test (Appendix VIII C), and specific precipitation lines shall be produced with soluble antigen of *Bacilla anthracis*.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless or light yellow liquid, free from foreign matters. After storage for a long time, a trace of precipitate may occur, which can be dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.3 Sodium chloride content

The Sodium chloride content shall be 7.5-9.5 g/L

(Appendix VII G).

3.3.3.4 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.5 Preservative content

If any of the following preservatives is added, it shall be:

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

The F(ab)₂ content shall be not less than 60% (Appendix VIII F).

3.3.5 Potency test

See Section 3.1.2.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package insert

The Requirements for Packaging of Biologics shall apply.

Rabies Antiserum

Rabies antiserum is a liquid preparation containing anti-rabies globulins. It is obtained by purification following pepsin digestion from the plasma of horses that have been immunized with fixed rabies virus. The preparation is used in combination with

the rabies vaccine to prevent rabies.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Antigen and adjuvant

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply.

2.2 Animals used for immunization and plasma

2.2.1 Animals used for immunization

The horses used for immunization must comply with the Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera.

2.2.2 Immunization, blood collection and plasma separation

The Requirements for Quarantine and Immunization of Horses Used for Production of Immunosera shall apply. Blood shall be collected when the potency of immunoserum is not less than 100 IU/ml. A quantity of appropriate preservative may be added to plasma and sterility test shall be carried out (Appendix XIII A).

2.3 Bulk

2.3.1 Source plasma

The neutralizing potency of source plasma shall be not less than 80 IU/ml (Appendix XII J). If apparent hemolysis, bacterial contamination or other abnormalities occur in the plasma during storage, the plasma shall not be used for production.

2.3.2 Preparation

2.3.2.1 Digestion

Add a quantity of pepsin and toluene to the diluted plasma, and digest at a proper pH and a suitable temperature for a certain period of time.

2.3.2.2 Purification

The above digest shall be purified by sequential steps of heating, ammonium sulfate fractionation, alum adsorption, etc.

2.3.2.3 Concentration, clarification and sterilization by filtration

The bulk may be concentrated by ultrafiltration or ammonium sulfate precipitation. After clarification and sterilization by filtration, a quantity of trichloromethane, thimerosal or metacresol may be added to the preparation as a preservative.

The purified bulk antiserum shall be stored at 2-8°C and protected from light for at least one month as a stabilizing period.

2.3.3 Control tests on bulk

See Section 3.1.

2.4 Final bulk

2.4.1 Formulation

Dilute the bulk qualified in control tests with sterile water for injection, adjust the titer, protein content, pH and sodium chloride content according to the specifications of the final products, and sterilize the bulk by filtration.

2.4.2 Control tests on final bulk

See Section 3.2.

2.5 Final product

2.5.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.5.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.5.3 Specifications

Not less than 400 IU of the titer of rabies antiserum per container.

2.5.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.1.2 Antibody potency

Carry out the test for potency (Appendix XI J).

3.1.3 Sterility test

It complies with the test for sterility (Appendix XII A).

3.1.4 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

At least one container from each final batch shall be sampled for identity test.

3.3.1.1 Neutralization test in animals

Perform the test for identity as specified in Appendix XI J. The result shall demonstrate that the preparation can neutralize rabies virus in animals.

3.3.1.2 Double immunodiffusion test

Perform the double immunodiffusion test (Appendix VIII C), and precipitation lines shall be produced only with anti-horse serum.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless or

light yellow liquid, free from foreign matters. After storage for a long time, a trace of precipitate may occur, which can be dispersed on shaking.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.0-7.0 (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 170 g/L (Appendix VI B, method 1).

3.3.3.3 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.3.4 Ammonium sulfate content

The ammonium sulfate content shall be not more than 1.0 g/L (Appendix VII C).

3.3.3.5 Preservative content

If any of the following preservatives is added, it shall be;

not more than 0.1 g/L for the content of thimerosal (Appendix VII B);

not more than 2.5 g/L for the content of meta-cresol (Appendix VI N);

not more than 0.5% for the content of trichloromethane (Appendix VI O).

3.3.4 Purity

3.3.4.1 Albumin detection

Dilute sample to a protein content of 2% and carry out agarose electrophoresis (Appendix IV B). The result shall reveal the absence of protein or only a trace of protein with the migrating rate of albumin.

3.3.4.2 F(ab)₂ content

It shall be not less than 60% (Appendix VIII F).

3.3.5 Antibody potency

The titer of rabies antiserum shall be not less than 200 IU/ml (Appendix XI J). The filling quantity in each container shall be not less than the stated value.

3.3.6 Content of blood group A-like substance

The content of blood group A-like substance shall be not more than 4 µg/ml (Appendix IX I).

3.3.7 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the preparation shall be 3.0 ml/kg of rabbit body weight.

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Albumin

Human albumin is a liquid preparation made from plasma of healthy donors by cold ethanol fractionation or other approved methods and heated at 60°C for 10 hours for viral inactivation. The preparation contains a suitable stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with the General Requirements for Source Plasma of Blood Products.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The quality of fraction IV used as source materials shall comply with the standards given in the annex of this monograph.

2.1.4 Fraction IV shall be stored at -30°C or below and shipped at -15°C or below. The storage period of frozen fraction IV shall not exceed 12 months.

2.1.5 Fraction V shall be stored at -30°C or below and the period for storage shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production process. When fraction IV is used as source materials, cold ethanol fractionation combined with column chromatography can be used.

2.2.2 Fraction V after purification, ultrafiltration and sterilization by filtration is regarded as the bulk albumin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the product on the basis of one gram of protein with 0.16 mmol of sodium caprylate or 0.08 mmol of sodium caprylate and 0.08 mmol of sodium acetyltryptophan. The protein concentration shall

be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Viral inactivation

The product shall be heated in a water bath of $60^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ continuously for at least 10 hours to inactivate potentially existing residual viruses. Viral inactivation can be performed before or within 24 hours after sterilization by filtration and filling.

2.3.3 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Incubation

The preparation after filling shall be kept at $20-25^{\circ}\text{C}$ for at least 4 weeks, or at $30-32^{\circ}\text{C}$ for at least 14 days. Visual inspection on each container shall be carried out. Sterility test shall be performed when turbidity or precipitate is shown. Those unqualified in the sterility test shall not be reused for production.

2.4.4 Specifications

2 g, 5 g, 10 g or 12.5 g of protein per container, with the protein concentrations of 5%, 10%, 20% and 25%, respectively.

2.4.5 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be more than the stated value of final product.

3.1.2 Purity

Albumin shall be not less than 96.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix VIII A). If the final bulk is to be filled immediately after formulation, the samples for sterility tests shall be taken after sterilization by filtration.

3.2.2 Pyrogen test

It complies with the test for Appendix VIII D. The injecting dose of the product shall be 0.6 g/kg of rabbit body weight. Alternatively, the test for bacterial endotoxin (Appendix VIII E) shall apply. The limit value (L) of bacterial endotoxin shall be less than 0.5 EU/ml, 0.83 EU/ml, 1.67 EU/ml and 2.08 EU/ml when the protein contents are 5%, 10%, 20% and 25%, respectively.

3.3 Control tests on final product

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be albumin as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, slightly viscous liquid without turbidity, slightly yellow, green or brown in colour.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the Requirements for Filling Quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.2.4 Thermostability test

Keep the products in a water bath of $57^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 50 hours, no visual changes shall appear except slight change in colour in comparison with that of the unheated product of the same batch.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not less than 95.0% of the stated value (Appendix VI B, method 1).

3.3.3.3 Purity

Albumin shall be not less than 96.0% of the total protein (Appendix IV A).

3.3.3.4 Sodium content

The sodium content shall be not more than 160 mmol/L (Appendix VII J).

3.3.3.5 Potassium content

The potassium content shall be not more than 2 mmol/L (Appendix VII I).

3.3.3.6 Absorbance

When the product is diluted to the protein concentration of 10 g/L with physiological saline, the absorbance shall be not more than 0.15 when measured by spectrophotometer at 403 nm (Appendix II A).

3.3.3.7 Polymer content

The polymer content shall be not more than 5.0% (Appendix VI Q).

3.3.3.8 Sodium caprylate content

The sodium caprylate content shall be 0.140-0.180 mmol/g of protein. If sodium caprylate and sodium acetyltryptophan are used together, the content of sodium caprylate shall be 0.064-0.096 mmol/g of protein (Appendix VI K).

3.3.3.9 Residual aluminum content

The residual aluminum content shall be not more than 200 µg/L (Appendix VII K).

3.3.4 Prekallikrein activator (PKA)

The product made from fraction IV shall be tested for PKA content (Appendix IX F). The PKA shall be not more than 35.0 IU/ml.

3.3.5 HBsAg

Carry out the test for HBsAg according to the instructions of diagnostic kit used. The result shall be negative.

3.3.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.6 g/kg of rabbit body weight.

4 Storage, shipping and validity period

Store and ship at 2-8°C or at room temperature, protected from light. The approved validity period shall apply, starting from the date of filling of final product. Only one kind of storage temperature and validity period shall be prescribed on the label.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

6 Annex

Quality Standards of Fraction IV Used as Source Materials

**Quality Standards of Fraction IV
Used as Source Materials**

1 Fraction IV is one of plasma fractions prepared by cold ethanol fractionation. The source plasma used shall comply with the Requirements for Source Plasma of Blood Products.

2 Fraction IV shall be kept in such a way as to minimize the risk of contamination and stored frozen at or below -30°C. The storage period shall not exceed 12 months.

3 Control tests on fraction IV

Weigh accurately 10 g of fraction IV precipitate and dissolve in physiological saline to make a volume of 100 ml by stirring at 1-3°C. Carry out centrifugation or filtration and take the supernatant for the following tests.

3.1 Identity test**3.1.1 Double immunodiffusion**

Perform the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-sheep or anti-pig serum.

3.1.2 Immunoelectrophoresis

Perform the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be albumin in comparison with normal human serum.

3.2 Protein content

The protein content shall be not less than 2.5% (Appendix VI B, method 3).

3.3 Albumin purity

The albumin shall be not less than 20% of total protein (Appendix IV A, method 3).

3.4 HBsAg

Carry out the test for HBsAg according to the instructions of diagnostic kit used. The result shall be negative.

3.5 HIV-1/HIV-2 antibodies

Carry out the test for HIV-1/HIV-2 antibodies according to the instructions of diagnostic kit used. The result shall be negative.

3.6 HCV antibodies

Carry out the test for HCV according to the instructions of diagnostic kit used. The result shall be negative.

3.7 Counting of culture colonies

The test samples shall be taken in triplicate. Add 1 ml of supernatant of each sample onto 9 ml of agar medium of trypticase and soybean extract, and incubate at 32-35°C for 72 hours. The result shall be not more than 50 CFU/ml of supernatant on average.

Human Albumin, Freeze-dried

Freeze-dried human albumin is a preparation made from plasma of healthy donors by cold ethanol fractionation or other approved methods and heated at 60°C for 10 hours for viral inactivation. The preparation contains a suitable stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with the General Requirements for Source Plasma of Blood Products.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The quality of fraction IV used as source materials shall comply with the standards given in the annex of the requirements for Human Albumin.

2.1.4 Fraction IV shall be stored at -30°C or below and shipped at -15°C or below. The storage period of frozen fraction IV shall not exceed 12 months.

2.1.5 Fraction V shall be stored at -30°C or below and the period for storage shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production process. When fraction IV is used as source materials, cold ethanol fractionation combined with column chromatography can be used.

2.2.2 Fraction V after purification, ultrafiltration and sterilization by filtration is regarded as the bulk albumin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the product on the basis of one gram of protein with 0.16 mmol of sodium caprylate or 0.08 mmol of sodium caprylate and 0.08 mmol of sodium acetyltryptophan. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly

adjusted.

2.3.2 Viral inactivation

The product shall be heated in a water bath of 60°C ± 0.5°C continuously for at least 10 hours to inactivate potentially existing residual viruses. Viral inactivation can be performed before or within 24 hours after sterilization by filtration and filling.

2.3.3 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 50°C. The final containers shall be sealed under vacuum.

2.4.3 Specifications

2 g, 5 g, 10 g or 12.5 g of protein per container, with protein concentrations of 5%, 10%, 20% and 25% respectively.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be more than the stated value of final product.

3.1.2 Purity

Albumin shall be not less than 96.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix VII A). If the final bulk is to be filled immediately after formulation, the samples for sterility tests shall be taken after sterilization by filtration.

3.2.2 Pyrogen test

It complies with the test for pyrogen (Appendix VIII D). The injecting dose of the product shall be 0.6 g/kg of rabbit body weight. Alternatively, the test for bacterial endotoxin (Appendix VIII E) shall apply. The limit value (L) of bacterial endotoxin shall be less than 0.5 EU/ml, 0.83 EU/ml, 1.67 EU/ml and 2.08 EU/ml when the protein contents are 5%, 10%, 20% and 25%, respectively.

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be albumin as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-white crisp cake without any sign of thawing. The reconstituted product shall be a clear, slightly viscous liquid without turbidity, slightly yellow, green or brown in colour.

3.3.2.2 Vacuum detection

The product shall be tested with a high frequency spark vacuum detector and the blue-purple glow shall be seen in the final container.

3.3.2.3 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.4 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.5 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 1.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with

physiological saline (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not less than 95.0% of the stated value (Appendix VI B, method 1).

3.3.3.4 Purity

Albumin shall be not less than 96.0% of the total protein (Appendix IV A).

3.3.3.5 Sodium content

The sodium content shall be not more than 160 mmol/L (Appendix VII J).

3.3.3.6 Potassium content

The potassium content shall be not more than 2 mmol/L (Appendix VII I).

3.3.3.7 Absorbance

When the product is diluted to the protein concentration of 10 g/L with physiological saline, the absorbance shall be not more than 0.15 when measured by spectrophotometer at 403 nm (Appendix II A).

3.3.3.8 Polymer content

The polymer content shall be not more than 5.0% (Appendix VI Q).

3.3.3.9 Sodium caprylate content

The sodium caprylate content shall be 0.140-0.180 mmol/g of protein. If sodium caprylate and sodium acetyltryptophan are used together, the content of sodium caprylate shall be 0.064-0.096 mmol/g of protein (Appendix VI K).

3.3.3.10 Residual aluminum content

The residual aluminum content shall be not more than 200 µg/L (Appendix VII K).

3.3.4 Prekallikrein activator (PKA)

The product made from fraction IV shall be tested for PKA content (Appendix IX F). The PKA content shall be not more than 35.0 IU/ml.

3.3.5 HBsAg

Carry out the test for HBsAg according to the instructions of diagnostic kit used. The result shall be negative.

3.3.6 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix VIII D). The injecting dose of the product shall be 0.6 g/kg of rabbit body weight.

4 Storage, shipping and validity period

Store and ship at or below 8°C or at room temperature, protected from light. The approved validity period shall apply, starting from the date of filling of final product. Only one kind of storage temperature and validity period shall be prescribed on the label.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Immunoglobulin

Human immunoglobulin is a liquid preparation made from plasma of healthy donors by cold ethanol fractionation or other approved methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing**2.1 Source plasma**

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 100 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C , and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk**2.3.1 Formulation**

A quantity of stabilizer shall be added to the preparation and thimerosal may be used as a preservative. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product**2.4.1 Defining batches**

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

150 mg or 300 mg of protein per container, with protein concentration of 10%.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests**3.1 Control tests on bulk****3.1.1 Protein content**

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be more than the stated value of final product.

3.1.2 Purity

The immunoglobulin (Ig G) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Pyrogen test

It complies with the test for pyrogen (Appendix VIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk**Sterility test**

It complies with the test for sterility (Appendix VIII A).

3.3 Control tests on final product**3.3.1 Identity test****3.3.1.1 Double immunodiffusion**

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immuno-electrophoresis

Carry out the test for identity by immuno-electrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human



serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.2.4 Thermostability test

Keep the products in a water bath of $57^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 4 hours, no gelation or flocculi shall appear.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not less than 95.0% of the stated value (Appendix VI B, method 1).

3.3.3.3 Purity

Immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.4 Saccharide content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content shall be not more than 50 g/L (Appendix VI P).

3.3.3.5 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.6 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Potency test

3.3.4.1 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA kit used. The anti-HBs potency shall be not less than 6.0 IU/g of protein.

3.3.4.2 Diphtheria antibody

The diphtheria antibody shall be not less than 3.0 HAU/g of protein (Appendix X O).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Immunoglobulin, Freeze-dried

Freeze-dried human immunoglobulin is a preparation made from plasma of healthy individual by cold ethanol fractionation or other approved methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 1000 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C , and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the

preparation and thimerosal may be used as a preservative. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk
See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum.

2.4.3 Specifications

150 mg or 300 mg of protein per container, with protein concentration of 10%.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be more than the stated value of final product.

3.1.2 Purity

The immunoglobulin (Ig G) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight. The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-white crisp cake without any sign of thawing. The reconstituted product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.3 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.4 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not less than 95.0% of the stated value (Appendix VI B, method 1).

3.3.3.4 Purity

Immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.5 Saccharide content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content shall be not more than 50 g/L (Appendix VI P).

3.3.3.6 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.7 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Potency test

3.3.4.1 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA kit used. The anti-HBs potency shall be not less than 6.0 IU/g of protein.

3.3.4.2 Diphtheria antibody

The diphtheria antibody shall be not less than 3.0 HAU/g of protein (Appendix X O).

3.3.5 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix VIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Hepatitis B Immunoglobulin

Human hepatitis B immunoglobulin is a liquid preparation made from pooled anti-HBs-rich plasma of healthy donors immunized with hepatitis B vaccine, by cold ethanol fractionation or other approved methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the require-

ments set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products. The licensed hepatitis B vaccine used as an antigen and the approved immunization schedule shall apply. The anti-HBs potency in the pooled plasma shall be not less than 8 IU/ml.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 100 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C, and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk
See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the preparation and thimerosal may be used as a preservative. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk
See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

100 IU, 200 IU or 400 IU of anti-HBs per container, containing not less than 100 IU/ml of anti-HBs.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not more than 180 g/L.

3.1.2 Purity

The immunoglobulin (Ig G) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.1.6 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA Kit used. It shall be not less than the specification of the final product. The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immuno-electrophoresis

Carry out the test for identity by immuno-electrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without

turbidity.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A).

3.3.2.4 Thermostability test

Keep the products in a water bath of $57^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 4 hours. No gelation or flocculi shall appear.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 180 g/L (Appendix VI B, method 1).

3.3.3.3 Purity

Immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.4 Saccharides content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content shall be not more than 50 g/L (Appendix VI P).

3.3.3.5 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.6 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA kit used. The anti-HBs potency shall be not less than 100 IU/ml. The anti-HBs potency of each container shall be not less than the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting

from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Hepatitis B Immunoglobulin, Freeze-dried

Freeze-dried human hepatitis B immunoglobulin is a preparation made from pooled anti-HBs-rich plasma of healthy donors immunized with hepatitis B vaccine, by cold ethanol fractionation or other approved methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products. The licensed hepatitis B vaccine used as an antigen and the approved immunization schedule shall apply. The anti-HBs potency in the pooled plasma shall be not less than 8 IU/ml.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 100 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C , and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the preparation and thimerosal may be used as a preservative. The protein concentration shall be

adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C . The final containers shall be sealed under vacuum.

2.4.3 Specifications

100 IU, 200 IU or 400 IU of anti-HBs per container, containing not less than 100 IU/ml of anti-HBs.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not more than 180 g/L.

3.1.2 Purity

The immunoglobulin (IgG) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Pyrogen test

It complies with the test for pyrogen (Appendix VIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.1.6 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA Kit used. It shall be not less than the specification of the final product.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content, and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immuno-electrophoresis

Carry out the test for identity by immuno-electrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-white crisp cake without any sign of thawing. The reconstituted product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.3 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.4 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VIII D).

3.3.3.2 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 180 g/L (Appendix VI B, method 1).

3.3.3.4 Purity

Immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.5 Saccharide content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content shall be not more than 50 g/L (Appendix VI P).

3.3.3.6 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.7 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA kit used. The anti-HBs potency shall be not less than 100 IU/ml. The anti-HBs potency of each container shall be not less than the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Rabies Immunoglobulin

Human rabies immunoglobulin is a liquid preparation made from pooled rabies antibody-rich plasma of healthy donors immunized with rabies vaccine by cold ethanol fractionation or other approved separation methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the require-

ments set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products. The licensed rabies vaccine used as an antigen and the approved immunization schedule shall apply. The blood sample from immunized donors shall be tested for antibody titer with ELISA, plaque formation test or neutralization test in brains of mice. Once the rabies antibody reaches 10 IU/ml after immunization, the plasma can be collected.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 100 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at -30°C or below, and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the preparation and thimerosal may be used as a preservative. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

100 IU, 200 IU or 500 IU of rabies antibody per container, containing not less than 100 IU/ml of rabies antibody.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not more than 180 g/L.

3.1.2 Purity

The immunoglobulin (IgG) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Rabies antibody potency

It shall be not less than the stated value of final product. (Appendix XI J).

3.1.6 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, colourless or light

yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A).

3.3.2.4 Thermostability test

Keep the products in a water bath of $57^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 4 hours, no gelation or flocculi shall appear.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 180 g/L (Appendix VI B, method 1).

3.3.3.3 Purity

The immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.4 Saccharide content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content shall be not more than 50 g/L (Appendix VI P).

3.3.3.5 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.6 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Rabies antibody potency

It shall be not less than 100 IU/ml (Appendix XI J). The rabies antibody potency of each container shall be not less than the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Rabies Immunoglobulin, Freeze-dried

Freeze-dried human rabies immunoglobulin is a preparation made from pooled rabies antibody-rich plasma of healthy donors immunized with rabies vaccine, by cold ethanol fractionation or other approved separation methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products. The licensed rabies vaccine used as an antigen and the approved immunization schedule shall apply. The blood sample from immunized donors shall be tested for antibody titer with ELISA, plaque formation test or neutralization test in brains of mice. Once the rabies antibody reaches 10 IU/ml after immunization, the plasma can be collected.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 100 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C , and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the



preparation and thimerosal may be used as a preservative. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches for Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization for Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum.

2.4.3 Specifications

100 IU, 200 IU or 500 IU of rabies antibody per container, containing not less than 100 IU/ml of rabies antibody.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not more than 180 g/L.

3.1.2 Purity

The immunoglobulin (IgG) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Rabies antibody potency

It shall be not less than the stated value of final product (Appendix XI J).

3.1.6 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be

0.15 g/kg of rabbit body weight.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-white crisp cake without any sign of thawing. The reconstituted product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.3 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.4 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 180 g/L (Appendix VI B, method 1).

3.3.3.4 Purity

Immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.5 Saccharide content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content shall be not more than 50 g/L (Appendix VI P).

3.3.3.6 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.7 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Rabies antibody potency

It shall be not less than 100 IU/ml (Appendix XI J). The rabies antibody potency of each container shall be not less than the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Tetanus Immunoglobulin

Human tetanus immunoglobulin is a liquid preparation made from pooled tetanus antibody-rich plasma of healthy donors immunized with tetanus vaccine, by cold ethanol fractionation or other approved separation methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing**2.1 Source plasma**

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products. The licensed tetanus vaccine used as an antigen and the approved immunization schedule shall apply. The blood sample from immunized donors shall be tested for antibody titer. Once the tetanus antibody reaches 8 IU/ml after immunization, the plasma can be collected.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 100 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C, and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk**2.3.1 Formulation**

A quantity of stabilizer shall be added to the preparation and thimerosal may be used as a preservative. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product**2.4.1 Defining batches**

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

250 IU of tetanus antibody per container, containing not less than 100 IU/ml of tetanus antibody.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes

shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not more than 180 g/L.

3.1.2 Purity

The immunoglobulin (IgG) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.1.6 Tetanus antibody potency

It shall be not less than the stated value of final product (Appendix XI F). The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be

dispersed on shaking is allowable.

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A).

3.3.2.4 Thermostability test

Keep the products in a water bath of $57^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 4 hours, no gelation or flocculi shall appear.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.2 Protein content

The protein content shall be not more than 180 g/L (Appendix VI B, method 1).

3.3.3.3 Purity

Immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.4 Saccharide content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content shall be not more than 50 g/L (Appendix VI P).

3.3.3.5 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.6 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Tetanus antibody potency

It shall be not less than 100 IU/ml (Appendix XI F). The tetanus antibody potency of each container shall be not less than the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Tetanus Immunoglobulin, Freeze-dried

Freeze-dried human tetanus immunoglobulin is a preparation made from pooled tetanus antibody-rich plasma of healthy donors immunized with tetanus vaccine, by cold ethanol fractionation or other approved separation methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of antibiotics. Thimerosal may be added as a preservative.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products. The licensed tetanus vaccine used as an antigen and the approved immunization schedule shall apply. The blood sample from immunized donors shall be tested for antibody titer. Once the tetanus antibody reaches 8 IU/ml after immunization, the plasma can be collected.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 100 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C , and the storage period shall be defined.

2.2 Bulk

2.2.1 Cold ethanol fractionation or other approved methods shall be employed. Preservatives or antibiotics shall not be used in the production of bulk.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of stabilizer shall be added to the preparation and thimerosal may be used as a preservative. The protein concentration shall be adjusted according to the specifications of the final product by dilution with water for injection. pH

and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C . The final containers shall be sealed under vacuum.

2.4.3 Specifications

250 IU of tetanus antibody per container, containing not less than 100 IU/ml of tetanus antibody.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not more than 180 g/L.

3.1.2 Purity

The immunoglobulin (IgG) content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Pyrogen test

It complies with the test for pyrogen (Appendix VIII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.1.6 Tetanus antibody potency

It shall be not less than the stated value of final product (Appendix XI F).

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test



It complies with the test for sterility (Appendix VIII A).

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content, and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-white crisp cake without any sign of thawing. The reconstituted product shall be a clear liquid, colourless or light yellow in colour. Opalescence may occur but without turbidity.

3.3.2.2 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.3 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.4 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.3 Protein content

The protein content shall be not more than 180 g/L (Appendix VI B, method 1).

3.3.3.4 Purity

Immunoglobulin shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.5 Saccharide content

If any carbohydrate (glucose, maltose, etc) is used in the product as a stabilizer, the content

shall be not more than 50 g/L (Appendix VI P).

3.3.3.6 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.7 Thimerosal content

If thimerosal is used in the product as a preservative, the content shall be not more than 0.1 g/L (Appendix VII B).

3.3.4 Tetanus antibody potency

It shall be not less than 100 IU/ml (Appendix XI F). The tetanus antibody potency of each container shall be not less than the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

3.3.7 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.15 g/kg of rabbit body weight.

3.3.8 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Immunoglobulin (pH 4) for Intravenous Injection

Human immunoglobulin for intravenous injection is a liquid preparation made from plasma of healthy individual by cold ethanol fractionation or other approved methods, followed by the processes to remove anticomplement activity and to inactivate viruses. The preparation contains a suitable stabilizer, but free of preservative and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 1000 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C , and the storage period shall be defined.

2.2 Bulk

2.2.1 The cold ethanol fractionation or other approved separation methods shall be adopted for bulk preparation. The product shall have a defined distribution of IgG subclasses, which is similar to that of normal human serum, as the following reference data: 60.3%-71.5% for IgG₁; 19.4%-31.0% for IgG₂; 5.0%-8.4% for IgG₃ and 0.7%-4.2% for IgG₄. The Fc function of IgG shall be kept.

Preservatives or antibiotics shall not be used in the production process.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

The final bulk shall be formulated based on the specifications of final product containing not less than 50 g/L of IgG. The preparation may contain a quantity of maltose or other approved stabilizers.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

1 g, 1.25 g, 1.5 g, 2.5 g, 5 g or 10 g of IgG per container, containing 5% of IgG.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 IgG content

The IgG content shall be more than that of the anticipated (Appendix XI K).

3.1.2 Purity

The immunoglobulin content shall be not less than 95.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 3.8-4.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Anticomplement activity

The anticomplement activity shall be not more than 50% (Appendix IX K).

3.1.6 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 0.45 g of IgG/kg of rabbit body weight.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A). If the final bulk is to be filled immediately after formulation, the test sample shall be taken after sterilization by filtration.

3.3 Control tests on final product

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.2.4 Thermostability test

Keep the products in a water bath of $57^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 4 hours, no gelation or flocculi shall appear.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 3.8-4.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.2 IgG content

The protein content shall be not less than 90.0% of the stated value (Appendix XI K).

3.3.3.3 Purity

Immunoglobulin shall be not less than 95.0% of the total protein (Appendix IV A).

3.3.3.4 Saccharide and sugar alcohol contents

If any maltose or sucrose is added, the content shall be 90-110 g/L, if sorbitol or glucose is added, the content shall be 40-60 g/L (Appendix VI P).

3.3.3.5 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 95.0% of the total area of the chromatogram (Appendix VI R).

3.3.4 Potency test

3.3.4.1 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA kit used. The anti-HBs potency shall be not less than 6.0 IU/g of IgG.

3.3.4.2 Diphtheria antibody

The diphtheria antibody titer shall be not less than 3.0 HAU/g of IgG (Appendix X O).

3.3.5 Prekallikrein activator

The prekallikrein activator content shall be not more than 35.0 IU/ml (Appendix IX F).

3.3.6 Anticomplement activity

The anticomplement activity shall be not more than 50% (Appendix IX K).

3.3.7 Anti-A and anti-B hemagglutinins

The anti-A and anti-B hemagglutinin titers shall be not more than 1 : 64 (Appendix IX J).

3.3.8 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.45 g of IgG/kg of rabbit body weight.

3.3.11 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting

from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Immunoglobulin (pH 4) for Intravenous Injection, Freeze-dried

Freeze-dried human immunoglobulin for intravenous injection is a preparation made from plasma of healthy individual by cold ethanol fractionation or other approved methods, followed by the processes to remove anticomplement activity and to inactivate viruses. The preparation contains a suitable stabilizer, but free of preservative and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 1000 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at or below -30°C , and the storage period shall be defined.

2.2 Bulk

2.2.1 The cold ethanol fractionation or other approved separation methods shall be adopted for bulk preparation. The product shall have a defined distribution of IgG subclasses, which is similar to that of normal human serum, as the following reference data: 60.3%-71.5% for IgG₁; 19.4%-31.0% for IgG₂; 5.0%-8.4% for IgG₃ and 0.7%-4.2% for IgG₄. The Fc function of IgG shall be kept.

Preservatives or antibiotics shall not be used in the production process.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

The final bulk shall be formulated based on the specifications of final product containing not less than 50 g/L of IgG. The preparation may contain a quantity of maltose or other approved stabilizers.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum.

2.4.3 Specifications

1 g, 1.25 g, 1.5 g, 2.5 g, 5 g or 10 g of IgG per container, containing 5% of IgG.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 IgG content

The IgG content shall be more than that of the anticipated (Appendix XI K).

3.1.2 Purity

The immunoglobulin content shall be not less than 95.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 3.8-4.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Anticomplement activity

The anticomplement activity shall be not more than 50% (Appendix IX K).

3.1.6 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.45 g of IgG/kg of rabbit body weight. The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A). If the final bulk is to be filled immediately after formulation, the test sample shall be taken after sterilization by filtration.

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content, and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-white crisp cake without any sign of thawing. The reconstituted product shall be a clear, colourless or light yellow liquid. Opalescence may occur but without turbidity.

3.3.2.2 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.3 Vacuum detection

The product shall be tested with a high frequency spark vacuum detector and the blue-purple glow shall be seen in the final container.

3.3.2.4 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.5 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 3.8-4.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.3 IgG content

The protein content shall be not less than 90.0% of the stated value (Appendix XI K).

3.3.3.4 Purity

Immunoglobulin shall be not less than 95.0% of the total protein (Appendix IV A).

3.3.3.5 Saccharide and sugar alcohol contents

If any maltose or sucrose is added, the content shall be 90-110 g/L, if sorbitol or glucose is added the content shall be 40-60 g/L (Appendix VI P).

3.3.3.6 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 95.0% of the total area of the chromatogram (Appendix VI R).

3.3.4 Antibody**3.3.4.1 Anti-HBs potency**

Carry out the test for anti-HBs potency according to the instructions of RIA kit used. The anti-HBs potency shall be not less than 6.0 IU/g of IgG.

3.3.4.2 Diphtheria antibody

The diphtheria antibody titer shall be not less than 3.0 HAU/g of IgG (Appendix X O).

3.3.5 Prekallikrein activator

The prekallikrein activator content shall be not more than 35.0 IU/ml (Appendix IX F).

3.3.6 Anticomplement activity

The anticomplement activity shall be not more than 50% (Appendix IX K).

3.3.7 Anti-A and anti-B hemagglutinins

The anti-A and anti-B hemagglutinin titers shall be not more than 1 : 64 (Appendix IX J).

3.3.8 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 0.45 g of IgG/kg of rabbit body weight.

3.3.11 Additional tests shall be performed depending on the methods used for virus inactivation.

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Immunoglobulin for Intravenous Injection

Human immunoglobulin for intravenous injection

is a freeze-dried preparation made from plasma of healthy individual by cold ethanol fractionation or other approved methods, followed by the processes to remove anticomplement activity and to inactivate viruses. The preparation contains a suitable stabilizer, but free of preservative and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing**2.1 Source plasma**

2.1.1 Collection and the quality of plasma shall comply with General Requirements for Source Plasma of Blood Products.

2.1.2 The storage period of frozen plasma shall not exceed 24 months.

2.1.3 The plasma used for production of one batch shall be pooled from at least 1000 donors.

2.1.4 The precipitate of fractions II + III or fractions I + II + III shall be stored frozen at -30°C or below, and the storage period shall be defined.

2.1.5 The albumin used as a stabilizer shall comply with the requirements for Human Albumin.

2.2 Bulk

2.2.1 The cold ethanol fractionation or other approved separation methods shall be adopted for bulk preparation. The product shall have a defined distribution IgG subclasses, which is similar to that of normal human serum, as the following reference data: 60.3%-71.5% for IgG₁; 19.4%-31.0% for IgG₂; 5.0%-8.4% for IgG₃ and 0.7%-4.2% for IgG₄. The Fc function of IgG shall be kept.

Preservatives or antibiotics shall not be used in the production process.

2.2.2 The fraction after purification, ultrafiltration and sterilization by filtration is regarded as the bulk immunoglobulin.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk**2.3.1 Formulation**

The final bulk shall be formulated based on the specifications of final product containing not less than 30 g/L of IgG. The preparation may contain a quantity of albumin and glucose or sucrose, or other approved stabilizers.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product**2.4.1 Defining batches**

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum.

2.4.3 Specifications

1 g, 1.25 g, 1.5 g, 2.5 g or 5 g of IgG per container.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 IgG content

The IgG content shall be more than that of the anticipated (Appendix XI K).

3.1.2 Purity

The immunoglobulin content shall be not less than 95.0% of the total protein (Appendix IV A).

3.1.3 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.4 Residual ethanol content

It may be determined by Conway method (Appendix VI D). The content of residual ethanol shall be not more than 0.025%.

3.1.5 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 95.0% of the total area of the chromatogram (Appendix VI R).

3.1.6 Anticomplement activity

The anticomplement activity shall be not more than 50% (Appendix IX K).

3.1.7 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product containing 50 g/L of protein shall be 10 ml/kg of rabbit body weight.

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A). If the final bulk is to be filled immediately after formulation, the test sample shall be taken after sterilization by filtration.

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content, and filling quantity, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be IgG as compared with normal human serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-white crisp cake without any sign of thawing. The reconstituted product shall be a clear liquid, colourless or light yellow in colour. Opalescence may occur but without turbidity.

3.3.2.2 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.3 Vacuum detection

The product shall be tested with a high frequency spark vacuum detector and the blue-purple glow shall be seen in the final container.

3.3.2.4 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.5 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.4-7.4 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.3 IgG content

The protein content shall be not less than 90.0% of the stated value (Appendix XI K).

3.3.3.4 Purity

The Immunoglobulin shall be not less than 95.0% of the total protein (Appendix IV A). (Zone of albumin shall be excluded in the calculation).

3.3.3.5 Saccharide content

If glucose is added, the content shall be 35-55 g/L; if sucrose is added, the content shall be 40-80 g/L (Appendix VI P).

3.3.3.6 Sodium content

The sodium content shall be not more than 160 mmol/L (Appendix VII J).

3.3.4 Antibody

3.3.4.1 Anti-HBs potency

Carry out the test for anti-HBs potency according to the instructions of RIA kit used. The anti-HBs potency shall be not less than 6.0 IU/g of protein.

3.3.4.2 Diphtheria antibody

The diphtheria antibody titer shall be not less than 3.0 HAU/g of protein (Appendix X O).

3.3.5 Prekallikrein activator

The prekallikrein activator content shall be not more than 35.0 IU/ml (Appendix IX F).

3.3.6 Anticomplement activity

The anticomplement activity shall be not more than 50% (Appendix IX K).

3.3.7 Anti-A and anti-B hemagglutinins

The anti-A and anti-B hemagglutinin titers shall be not more than 1 : 64 (Appendix IX J).

3.3.8 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.10 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product containing 50 g/L of protein shall be 10 ml/kg of rabbit body weight.

3.3.11 Additional control tests shall be performed depending on the methods used for virus inactivation. If tributylphosphate and polysorbate 80 are used, their residual content shall be tested.

3.3.11.1 Residual tributylphosphate content

The residual tributylphosphate content shall be not more than 10 µg/ml (Appendix VI J).

3.3.11.2 Residual polysorbate 80 content

The residual polysorbate 80 content shall be not more than 100 µg/ml (Appendix VI H).

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Coagulation Factor VIII

Human coagulation factor VIII is a freeze-dried preparation made from plasma of healthy individuals by separation and purification, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with the General Requirements for Source Plasma of Blood Products.

2.1.2 The plasma shall be free from clot, precipitate of fibrin, hemolysis, and lipid.

2.1.3 Measures shall be taken during the plasma collection so as to avoid injuring the skin and keep blood flow easily. Blood shall be mixed well with anticoagulant and collected in a plastic bag.

2.1.4 The storage period of frozen plasma shall not exceed 6 months.

2.2 Bulk

2.2.1 Human coagulation factor VIII shall be made with the approved process from frozen cryoprecipitate derived from fresh frozen plasma by separation and purification. Preservatives or antibiotics shall not be used in the production process.

2.2.2 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

It shall be formulated according to the specifications of the final product. A suitable stabilizer shall be added to preparation.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C. The final containers shall be sealed

under vacuum.

2.4.3 Specifications

50 IU, 100 IU, 200 IU, 250 IU, 300 IU, 400 IU, 500 IU or 1000 IU of factor VIII per container.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved process for removal and inactivation of lipid-enveloped virus or non-lipid-enveloped virus shall be employed in production. If viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 pH

The pH shall be 6.5-7.5 (Appendix V A).

3.1.2 Potency test

It complies with the test for potency (Appendix X N).

3.1.3 Specific activity

The specific activity shall be not less than 1.0 IU/mg of protein.

3.2 Control tests on final bulk

3.2.1 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 10 IU/kg of rabbit body weight.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final container

The product looks like a milky-white crisp cake. The reconstituted product shall be a clear, colourless liquid. Slight opalescence may occur.

3.3.2.2 Reconstitution time

The product shall be reconstituted with a volume of sterile water for injection at 25-37°C according to the stated value. It shall be reconstituted completely within 30 minutes on shaking gently.

3.3.2.3 Vacuum detection

The product shall be tested with a high frequency spark vacuum detector and the blue-purple glow shall be seen in the final container.

3.3.2.4 Test for visible particles

It complies with the test for visible particles (Appendix V B). A trace of slight protein particles is allowable.

3.3.2.5 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.5-7.5 (Appendix V A).

3.3.3.3 Sodium content

The sodium content shall be not more than 160 mmol/L (Appendix VII J).

3.3.3.4 Citrate content

If sodium citrate is used as stabilizer, the citrate content shall be not more than 25 mmol/L (Appendix VII H, method 1).

3.3.3.5 Residual polyethylene glycol content

The residual polyethylene glycol content shall be not more than 0.5 g/L when the product is prepared by PEG separation.

3.3.4 Potency test

Carry out the test for potency (Appendix X N). Based on the calculation of activity (IU/ml) of factor VIII and the stated value, the potency of factor VIII per container shall be 80%-140% of the stated value.

3.3.5 Specific activity

The specific activity shall be not less than 1.0 IU/mg protein. The test can be exempted if protein stabilizer is added to the product.

3.3.6 Anti-A and anti-B hemagglutinins

The anti-A and anti-B hemagglutinin titers shall be not more than 1 : 64 (Appendix IX J).

3.3.7 HBsAg

Carry out the test for HBsAg according to the instructions of diagnostic kit used. The result shall be negative.

3.3.8 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.9 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F). The injecting dose of the product shall be 15 IU per guinea pig and 1.5 IU per mouse.

3.3.10 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be

10 IU/kg of rabbit body weight.

3.3.11 Additional tests shall be performed according to the virus inactivation methods used. If tributylphosphate and polysorbate 80 are used, their residual contents shall be tested.

3.3.11.1 Residual tributylphosphate content

The residual tributylphosphate content shall be not more than 10 µg/mg (Appendix VI J).

3.3.11.2 Residual polysorbate 80 content

The residual polysorbate 80 content shall be not more than 100 µg/mg (Appendix VI H).

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Fibrinogen

Human fibrinogen is a freeze-dried preparation made from plasma of healthy donors by separation and purification, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with the General Requirements for Source Plasma of Blood Products.

2.1.2 Freshly collected liquid or frozen plasma, plasma after removal of cryoprecipitate and coagulation factors II, VII, IX and X may be used for production.

2.1.3 The storage period of frozen plasma shall not exceed 24 months.

2.2 Bulk

2.2.1 Cold ethanol fractionation shall be used for bulk preparation. Preservatives or antibiotics shall not be used in the production process.

2.2.2 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

The formulation shall be performed according to the specifications of the final product. A suitable

stabilizer can be added to the preparation.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum.

2.4.3 Specifications

0.5 g, 1.0 g, 1.5 g or 2.0 g of human fibrinogen per container.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved process for removal and inactivation of lipid-enveloped virus or non-lipid-enveloped virus shall be employed in production. If viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 pH

The pH shall be 6.5-7.5 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.1.2 Purity

The test sample is diluted with physiological saline to produce a fibrinogen solution of 2-3 mg/ml for determination of protein content [P (g/L)] (Appendix VI B, method 1).

Add 10 ml of the above solution to 10 ml of 3 IU/ml thrombin solution (containing 0.05 mmol/L calcium chloride), followed by centrifugation at 2500 r/min or filtration to separate the precipitate after incubating at 37°C for 20 minutes. After washing the precipitate with physiological saline for 3 times, the coagulated protein content [F (g/L)] shall be tested (Appendix VI B, method 1). The purity calculated by the following equation shall be not less than 70.0%.

$$\text{The purity of fibrinogen} = \frac{F \text{ (g/L)}}{P \text{ (g/L)}} \times 100\%$$

3.1.3 Coagulation activity

Add 0.5 ml of thrombin (3 IU/ml) to the test tubes and incubate in 37°C water bath of for one minute, and then mix 0.5 ml of test sample diluted to a concentration of 3 mg/ml with physiological saline. Maintain the mixture in 37°C water bath, record the clotting time. The mean

clotting time of the two tests shall be not more than 60 seconds.

3.2 Control tests on final bulk

3.2.1 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 30 mg/kg of rabbit body weight.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final container

The product looks like a greyish-white or light yellow crisp cake. The reconstituted product shall be a clear liquid. Slight opalescence may occur.

3.3.2.2 Vacuum detection

The product shall be tested with a high frequency spark vacuum detector and the blue-purple glow shall be seen in the final container.

3.3.2.3 Reconstitution time

The product shall be reconstituted with a volume of sterile water for injection at 30-37°C according to the stated value. It shall be reconstituted completely within 30 minutes on shaking gently.

3.3.2.4 Test for visible particles

It complies with the test for visible particles (Appendix V B). A trace of slight flocculi or protein particles is allowable.

3.3.2.5 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.2.6 Thermostability

Keep the reconstituted test sample in a water bath at 30-37°C for 60 minutes. No clot or precipitate of fibrin shall appear.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 5.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.5-7.5 when the product is diluted to the protein concentration of 10 g/L with physiological saline (Appendix V A).

3.3.3.3 Purity

See Section 3.1.2. It may be exempted when protein stabilizer is added to the product.

3.3.3.4 Total fibrinogen content

The total content of fibrinogen shall be not less than the stated value, based on the result in Section 3.3.3.3 and the stated value.

3.3.3.5 Citrate content

The citrate content shall be 39-54 mmol/L (Appendix VII H, method 1).

3.3.3.6 Saccharide content

If glucose or sucrose is added to the product, the saccharide content shall be 40-60 g/L (Appendix VI P).

3.3.3.7 Sodium chloride content

The sodium chloride content shall be 7.5-9.5 g/L (Appendix VII G).

3.3.4 Coagulation activity

See Section 3.1.3.

3.3.5 HBsAg

Carry out the test for HBsAg according to the instructions of the diagnostic kit. The result shall be negative.

3.3.6 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F). The product shall be diluted with physiological saline to a protein concentration of 10 g/L.

3.3.8 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 30 mg/kg of rabbit body weight.

3.3.9 Additional control tests shall be performed depending on the method used for virus inactivation. If tributylphosphate and polysorbate 80 are used, their residual contents shall be tested.

3.3.9.1 Residual tributylphosphate content

The residual tributylphosphate content shall be not more than 10 µg/ml (Appendix VI J).

3.3.9.2 Residual polysorbate 80 content

The residual polysorbate 80 content shall be not more than 100 µg/ml (Appendix VI H).

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Human Prothrombin Complex

Human prothrombin complex is a freeze-dried preparation made from plasma of healthy individuals by cold ethanol fractionation or other approved methods, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Collection and the quality of plasma shall comply with the Requirements for Source Plasma of Blood Products.

2.1.2 The plasma shall be free from clot, precipitate of fibrin, lipid and hemolysis.

2.1.3 The storage period of frozen plasma shall not exceed 24 months.

2.1.4 The plasma depleted of cryoprecipitate and coagulation factor VIII, fraction III precipitate may be used for production.

2.1.5 Fraction III precipitate shall be stored frozen at -30°C or below. The storage period shall not exceed 6 months.

2.2 Bulk

2.2.1 The bulk can be prepared by gel adsorption or cold ethanol and polyethylene glycol fractionation followed by gel adsorption, or by other approved methods. Preservatives or antibiotics shall not be used in the production process.

2.2.2 Control tests on bulk
See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

It shall be formulated according to the specifications of final product. A suitable stabilizer can be added to the preparation. If heparin is added, its content shall not exceed 0.5 IU per IU of factor IX.

2.3.2 Control tests on final bulk
See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of

Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C . The final containers shall be sealed under vacuum.

2.4.3 Specifications

100 IU, 200 IU, 300 IU, 400 IU or 1000 IU of factor IX per container together with variable amount of factors II, VII and X.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved process for removal and inactivation of lipid-enveloped virus or non-lipid-enveloped virus shall be employed in production. If viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 pH

The pH shall be 6.5-7.5 (Appendix V A)

3.1.2 Potency of factor IX

The potency of factor IX shall be not less than 10 IU/ml (Appendix X L).

3.1.3 Specific activity of factor IX

The specific activity shall be not less than 0.3 IU/mg of protein.

3.2 Control tests on final bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.2.2 Pyrogen test

It complies with the test for pyrogen (Appendix VIII A). The injecting dose of the product shall be 30 IU of factor IX/kg of rabbit body weight.

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-human serum, but no precipitation line with anti-horse, anti-bovine, anti-pig or anti-sheep serum.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white or greyish-green crisp cake. The reconstituted product shall be a clear liquid, colourless, light blue or yellowish green in colour.

3.3.2.2 Vacuum detection

The product shall be tested with a high frequency spark vacuum detector and the blue-purple glow shall be seen in the final container.

3.3.2.3 Reconstitution time

The product with a volume of sterile water for injection at 20-25°C according to the stated value. It shall be reconstituted completely within 15 minutes on shaking gently.

3.3.2.4 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.5 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical test

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.5-7.5 (Appendix V A).

3.3.3.3 Sodium content

It shall be not more than 160 mmol/L (Appendix VII J).

3.3.3.4 Citrate content

The citrate content shall be not more than 25 mmol/L (Appendix VII H, method 1).

3.3.3.5 Residual polyethylene glycol content

The residual polyethylene glycol content shall be not more than 0.5 g/L (Appendix VI G).

3.3.4 Potency test

3.3.4.1 Human coagulation factor IX

The potency of factor IX shall be not less than 10 IU/ml of the product (Appendix X L). The estimated potency of factor IX shall be not less than 80% and not more than 140% of the stated value based on the potency (IU/ml) of human coagulation factor IX and the stated value. The specific activity shall be not less than 0.3 IU/mg of protein.

3.3.4.2 Human coagulation factor II

The estimated potency shall be not less than 80% of the stated value, based on the potency (IU/ml) of human coagulation factor II (Appendix X J) and the stated value.

3.3.4.3 Human coagulation factor VII

The estimated potency shall be not less than 80% of the stated value, based on the potency (IU/ml) of human coagulation factor VII (Appendix X K) and the stated value.

3.3.4.4 Human coagulation factor X

The estimated potency shall be not less than 80% of the stated value, based on the potency (IU/ml) of human coagulation factor X (Appendix X M) and the stated value.

3.3.5 Human thrombin activity

Perform the test for human thrombin activity (Appendix IX N). No clot or precipitate of fibrin shall appear.

3.3.6 Heparin content

It shall be not more than 0.5 IU per IU of factor IX (Appendix IX P).

3.3.7 Activated coagulation factor

Clotting time shall be not less than 150 seconds (Appendix IX O).

3.3.8 HBsAg

Carry out the test for HBsAg according to the instructions of the diagnostic kit. The result shall be negative.

3.3.9 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.10 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F). Each guinea pig shall be injected with 5 ml of the test sample (containing 10 IU of factor IX/ml). Each mouse shall be injected with 0.5 ml of the test sample (containing 10 IU of factor IX/ml).

3.3.11 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose shall be 30 IU of factor IX/kg of rabbit body weight.

3.3.12 Additional control tests shall be performed depending on the methods used for virus inactivation. If tributylphosphate and polysorbate 80 are used, their residual contents shall be tested.

3.3.12.1 Residual tributylphosphate content

The residual tributylphosphate content shall be not more than 10 µg/ml (Appendix VI J).

3.3.12.2 Residual polysorbate 80 content

The residual polysorbate 80 content shall be not more than 100 µg/ml (Appendix VI H).

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Anti-human T Lymphocyte Porcine Immunoglobulin

Anti-human T lymphocyte porcine immunoglobulin is a liquid preparation made from plasma of pig immunized with human T lymphocyte by removal of other antibodies, purification and concentration, followed by viral inactivation. The preparation

contains a suitable stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Antigen used for immunization

The antigen used for immunization shall be the human thymocyte or human T lymphocyte isolated from the blood of healthy donors in accordance with the Requirements for Source Plasma of Blood Products. The thymocyte donor shall be serologically negative for HBsAg, anti-HCV, anti-HIV-1/HIV-2 and syphilis. The isolated T lymphocyte count and the erythrocyte count shall be not less than 90% and not more than 5% of the total cell count, respectively.

2.1.2 Animal used for immunization

The pig to be immunized shall be healthy and free from porcine pestis virus, porcine parvovirus, pseudorabies virus, foot-and-mouth disease virus and Japanese encephalitis virus. The body weight of the pig shall be 50-60 kg.

2.1.3 Immunization method

The approved immunization schedule shall apply.

2.1.4 Blood collection and plasma separation

After booster immunization the blood shall be collected when the titer determined by E-rosette forming inhibition test is not less than 1 : 1000. The separated plasma shall be stored at -20°C or below. The storage period shall not exceed 24 months.

2.2 Bulk

2.2.1 The titer of pooled plasma determined by E-rosette forming inhibition test shall be not less than 1 : 1000. The titer determined by lymphocytotoxicity test shall be not less than 1 : 500.

2.2.2 The plasma shall be inactivated in a water bath of 56°C for 30 minutes. The immunoglobulin shall be purified by means of absorption of other antibodies, ammonium sulfate precipitation and ion exchange chromatography.

The donor of human erythrocyte, human placental tissue and human plasma used to absorb other antibodies shall meet the donor's standards described in the General Requirements for Source Plasma of Blood Products.

2.2.3 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

A quantity of glycine shall be added to the preparation as a stabilizer. The protein concen-

tration shall be adjusted according to the specification of the final product by dilution with sterile water for injection. The pH and sodium chloride concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling of Biologics shall apply.

2.4.3 Specifications

250 mg of protein per container.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production process. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not less than 55 g/L.

3.1.2 Purity

The immunoglobulin content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.3 Human erythrocyte antibody

The human erythrocyte antibody titer shall be not more than 1 : 64 (Appendix IX Q).

3.1.4 Human platelet antibody

The human platelet antibody titer shall be not more than 1 : 4 (Appendix IX R).

3.1.5 Human plasma protein antibody

Carry out the test for human plasma protein antibody (Appendix VII C). Dilute the test sample and the positive control 2-fold serially to a dilution of 1 : 16 with physiological saline, respectively. Add the normal human plasma to the central well of a plate, while the different dilutions of test sample and the positive control to the peripheral wells. The test sample shall have no precipitation line with human plasma.

3.1.6 Potency test

3.1.6.1 E-rosette forming-inhibition test

The titer shall be not less than 1 : 4000 (Appendix X Q).

3.1.6.2 Lymphocytotoxicity test

The titer shall be not less than 1 : 1000 (Appendix X R).

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

3.2.1 Protein content

The protein content shall be 35-55 g/L (Appendix VI B, method 1).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.3 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). After the final bulk is diluted to a dilution of 1 : 4 with physiological saline, the injecting dose of the diluted product shall be 3 ml/kg of rabbit body weight.

3.3 Control tests on final product

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-porcine serum, but no precipitation line with anti-horse or anti-bovine serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be porcine IgG.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product is a clear liquid, light yellow in colour and opalescence may occur.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 (Appendix V A).

3.3.3.2 Total protein content

Perform the test for protein content (Appendix VI B, method 1). The total protein content shall be 175-275 mg per container, based on the protein content (g/ml) and the stated value.

3.3.3.3 Purity

The immunoglobulin content shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.4 Distribution of molecular size

The sum of IgG monomer and dimer shall be not

less than 90.0% of the total area of chromatogram while the IgG polymers shall be not more than 5.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.5 Ammonium sulfate content

The ammonium sulfate content shall be not more than 0.5 g/L (Appendix VII C).

3.3.3.6 Sodium chloride content

The sodium chloride content shall be 7-9 g/L (Appendix VII G).

3.3.4 Potency test

3.3.4.1 E-rosette forming-inhibition test

The titer shall be not less than 1 : 4000 (Appendix X Q).

3.3.4.2 Lymphocytotoxicity test

The titer shall be not less than 1 : 1000 (Appendix X R).

3.3.5 Human erythrocyte antibody

The human erythrocyte antibody titer shall be not more than 1 : 64 (Appendix IX Q).

3.3.6 Human platelet antibody

The human platelet antibody titer shall be not more than 1 : 4 (Appendix IX R).

3.3.7 Human plasma protein antibody

See Section 3.1.5.

3.3.8 Test for adventitious virus contamination

The test sample is subcultured continuously for three passages in a sensitive cell (such as BHK₂₁) for animal viruses. The result shall be negative.

3.3.9 HBsAg

Perform the test for HBsAg according to the instructions of the diagnostic kit used. The result shall be negative.

3.3.10 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.11 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F).

3.3.12 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). After the test sample is diluted to a dilution of 1 : 4 with physiological saline, the injecting dose of the dilution shall be 3 ml/kg of rabbit body weight.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Anti-human T Lymphocyte Rabbit Immunoglobulin

Anti-human T lymphocyte rabbit immunoglobulin is a freeze-dried preparation made from serum or plasma of rabbit immunized with human T lymphocyte by removal of other antibodies, purification and concentration, followed by viral inactivation. The preparation contains a suitable stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing

2.1 Source plasma

2.1.1 Antigen used for immunization

The antigen used for immunization shall be the human thymocyte or human T lymphocyte isolated from the blood of healthy donors in accordance with the Requirements for Source Plasma of Blood Products. The thymocyte donor shall be serologically negative for HBsAg, anti-HCV, anti-HIV-1/HIV-2 and syphilis. The isolated T lymphocyte count and the erythrocyte count shall be not less than 90% and not more than 5% of the total cell count, respectively.

2.1.2 Animal used for immunization

The rabbit each weighing 2-2.5 kg shall be used, which shall comply with the specified requirements in Appendices VIII B and VIII C.

2.1.3 Immunization method

The approved immunization schedule shall apply.

2.1.4 Blood collection and plasma separation

After booster immunization the blood shall be collected when the titer determined by lymphocytotoxicity test reaches 1 : 400. The separated plasma or serum shall be stored at -20°C or below. The storage period shall not exceed 24 months.

2.2 Bulk

2.2.1 The pooled plasma or serum shall be inactivated in a water bath of 56°C for 30 minutes. The immunoglobulin shall be purified by means of caprylic acid fractionation, ammonium sulfate precipitation, absorption of other antibodies, followed by DEAE-Sephadex A-50 chromatography. The donor of human erythrocyte, human placental tissue and human plasma used to absorb other antibodies shall meet the donor's standards described in the General Requirements for Source Plasma of Blood Products.

2.2.2 Control tests on bulk

See Section 3. 1.

2.3 Final bulk

2.3.1 Formulation

A quantity of maltose or other suitable stabilizer shall be added to the preparation. The protein concentration shall be adjusted according to the specification of the final product by dilution with sterile water for injection. The pH and sodium concentration shall be properly adjusted.

2.3.2 Control tests on final bulk

See Section 3. 2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization for Biologics shall apply. The filled preparation shall be frozen immediately after filling. The temperature of product during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum.

2.4.3 Specification

25 mg of protein per container.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

2.5 Viral removal and inactivation

The approved viral removal and inactivation processes shall be employed in production process. When viral inactivation agent (solvent, detergent) is used, the limit of the residual agent shall be defined.

3 Control tests

3.1 Control tests on bulk

3.1.1 Appearance

The preparation is a clear liquid, light orange-yellow in colour. Opalescence may occur, but free of foreign matters and precipitate.

3.1.2 Protein content

It may be determined with Biuret method (Appendix VI B, method 3). The protein content shall be not less than 10 g/L.

3.1.3 Purity

The immunoglobulin content shall be not less than 90.0% of the total protein (Appendix IV A).

3.1.4 Human erythrocyte antibody

The human erythrocyte antibody titer shall be not more than 1 : 64 (Appendix IX Q).

3.1.5 Human platelet antibody

The human platelet antibody titer shall be not more than 1 : 4 (Appendix IX R).

3.1.6 Human plasma protein antibody

Carry out the test for human plasma protein antibody (Appendix VIII C). Dilute the test sample and the positive control 2-fold serially to a dilution of 1 : 16 with physiological saline, respectively. Add the normal human plasma to the central well of a plate, while the different dilutions of test sample and the positive control to the peripheral wells. The test sample shall have no precipitation line with human plasma.

3.1.7 Potency test

3.1.7.1 E-rosette forming-inhibition test

The titer shall be not less than 1 : 512 (Appendix X Q).

3.1.7.2 Lymphocytotoxicity test

The titer shall be not less than 1 : 512 (Appendix X R).

The tests described above may be performed on final bulk.

3.2 Control tests on final bulk

3.2.1 Protein content

The protein content shall be not less than 1% (Appendix VI B, method 1).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.3 Pyrogen test

It complies with the test for pyrogen (Appendix XII D). The injecting dose of the product shall be 5 mg/kg of rabbit body weight.

3.3 Control tests on final product

Other than the tests for vacuum, reconstitution time, moisture content, and weight variation, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

3.3.1.1 Double immunodiffusion

Carry out the test for identity by double immunodiffusion (Appendix VIII C). There shall be only a precipitation line with anti-rabbit serum, but no precipitation line with anti-horse or anti-bovine serum.

3.3.1.2 Immunoelectrophoresis

Carry out the test for identity by immunoelectrophoresis (Appendix VIII D). The main precipitation line shall be rabbit IgG.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white crisp cake without any sign of thawing. The reconstituted product shall be a clear liquid, light orange-yellow in colour. Opalescence may occur.

3.3.2.2 Reconstitution time

The product is reconstituted with a volume of sterile water for injection at 20-30°C according to the stated value. It shall be reconstituted completely

within 15 minutes on shaking gently.

3.3.2.3 Test for visible particles

It complies with the test for visible particles (Appendix V B). The precipitate which can be dispersed on shaking is allowable.

3.3.2.4 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 3.8-4.4 (Appendix V A).

3.3.3.3 Total protein content

Perform the test for protein content (Appendix VI B, method 1). The total protein content shall be 20-30 mg per container, based on the protein content (g/ml) and the stated value.

3.3.3.4 Purity

The immunoglobulin content shall be not less than 90.0% of the total protein (Appendix IV A).

3.3.3.5 Maltose content

The maltose content shall be 20-30 g/L (Appendix VI P).

3.3.3.6 Distribution of molecular size

The sum of IgG monomer and dimer shall be not less than 90.0% of the total area of chromatogram while the IgG polymers shall be not more than 5.0% of the total area of the chromatogram (Appendix VI R).

3.3.3.7 Ammonium sulfate content

The ammonium sulfate content shall be not more than 0.5 g/L (Appendix VII C).

3.3.4 Potency test

3.3.4.1 E-rosette forming-inhibition test

The titer shall be not less than 1 : 512 (Appendix X Q).

3.3.4.2 Lymphocytotoxicity test

The titer shall be not less than 1 : 512 (Appendix X R).

3.3.5 Human erythrocyte antibody

The human erythrocyte antibody titer shall be not more than 1 : 64 (Appendix IX Q).

3.3.6 Human platelet antibody

The human platelet antibody titer shall be not more than 1 : 4 (Appendix IX R).

3.3.7 Human plasma protein antibody

See Section 3.1.6.

3.3.8 Test for adventitious virus contamination

The test sample is subcultured continuously for three passages in a sensitive cell (such as BHK₂₁) for animal viruses. The result shall be negative.

3.3.9 HBsAg

Perform the test for HBsAg according to the instructions of the diagnostic kit used. The result shall be negative.

3.3.10 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.11 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

3.3.12 Pyrogen test

It complies with the test for pyrogen (Appendix VIII D). The injecting dose of the product shall be 5 mg/kg of rabbit body weight.

4 Storage, shipping and validity period

Store and ship at 8°C or below, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Mouse Monoclonal Antibody Against Human CD3 Antigen of T Lymphocyte for Injection

The mouse monoclonal antibody against human CD3 antigen of T lymphocyte (MAb to CD3) for injection is a freeze-dried preparation made by using hybridoma technology. The monoclonal antibody against CD3 is prepared by purification of the MAb from ascitic fluid of the mice inoculated i. p. with MAb-secreting hybridoma cells and lyophilization of the purified MAb.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the relevant requirements set forth in the General Notices.

The mouse used for preparation of ascitic fluid shall be SPF BALB/c mice or F1 hybrid between the BALB/c strain and the Swiss strain.

2 Production

2.1 Hybridoma cell

Hybridoma cell, which secretes MAb to CD3 stably, shall comply with the Requirements for Preparations and Control Tests of Animal Cells as Substrates for Production of Biologics.

2.2 Establishment of hybridoma cell banks

Requirements for Preparations and Control Tests of Animal Cells as Substrates for Production of Biologics shall apply.

2.3 Control tests on hybridoma cell banks

The master and working cell banks shall be fully characterized according to the Requirements for

Preparations and Control Tests of Animal Cells as Substrates for Production of Biologics. In addition, the master cell bank is subject to the following control tests.

2.3.1 Karyotype

The chromosomes of one hundred cells in metaphase shall be examined. The karyotype of examined cells shall be characteristic of hybridoma cells of mouse-mouse.

2.3.2 Antibody-secreting stability

Hybridoma cells shall secrete specific antibody stably during passages *in vitro* for more than 3 months. Hybridoma cells stored in liquid nitrogen shall be able to be recovered and propagated, and shall secrete specific antibody stably.

2.3.3 Specificity

Specificity tests shall include test for molecular weights of antibody-specific antigens; reactivity with human tissues and cells such as thymus, tonsil, spleen and each component of human peripheral blood cells; reactivity with T and B leukemia cell lines. The specificity can also be evaluated by using the competitive assays with known specific MAbs. The results shall demonstrate that MAb is a specific antibody against human T lymphocyte CD3.

2.3.4 Types and subtypes of immunoglobulin

Types and subtypes of immunoglobulin shall be classified by double diffusion in agarose gel with anti-types and anti-subtypes of mouse immunoglobulin.

2.3.5 Affinity

It shall be not less than 10^7 L/mol.

2.3.6 Cross reactions

Cryotomy stain method shall be adopted. Cross reactions with thymus, tonsil, lymph nodes, spleen, bone marrow, blood cells, lung, liver, kidney, bladder, stomach, intestine, pancreas, parotid gland, thyroid, parathyroid, adrenal gland, hypophysis cerebri, peripheral nerve, ovary, testicle, skin and eye etc. shall be tested. MAb shall have no cross reactions with major human organ tissues and cells except target cells such as thymus, tonsil, lymph nodes, spleen, mesenteric lymph nodes and other lymph nodes.

2.4 Bulk

2.4.1 Preparation of ascitic fluid containing antibody

2.4.1.1 Resurrection and propagation of hybridoma cells from the working cell bank shall be carried out.

2.4.1.2 Hybridoma cells growing in log-phase are inoculated i. p. into mouse with 5×10^5 cells per mouse. Mice can be treated with Pristane or paraffin prior to inoculation with cells.

2.4.1.3 Mouse ascites are collected aseptically and stored at -20°C after processed appropriately.

2.4.1.4 Control tests on ascites

See Section 3.1.

2.4.2 Purification of antibody

2.4.2.1 Pre-treatment of ascites

Ascites collected in different days are pooled and filtered with filter paper.

2.4.2.2 Purification of IgG

The approved methods shall be used for the purification of IgG from ascites.

2.4.3 Processing after purification

2.4.3.1 Purified IgG is heat-inactivated at 56°C for 30 minutes, and then precipitates are removed by centrifugation.

2.4.3.2 Inactivation of viruses

The approved methods shall be used to remove and inactivate viruses.

2.4.3.3 Sterilization by filtration

Bulk IgG is prepared by sterilization by filtration.

2.4.4 Control tests on bulk

See Section 3.2.

2.5 Final bulk

2.5.1 Formulation

Bulk is diluted into a concentration of 5 mg/ml with 0.02 mol/L PBS. The stabilizer is added into the preparation and sterilization by filtration is followed.

2.5.2 Control tests on final bulk

See Section 3.3.

2.6 Final product

2.6.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.6.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.6.3 Specifications

1.0 ml per container after reconstitution, containing 5 mg of Mab to CD3.

2.6.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on ascites

3.1.1 Antibody activity

Reactivity with normal human peripheral blood mononuclear cells shall be tested by using immunofluorescent method. The reference value in normal range shall be $66.0\% \pm 10\%$ and the antibody titer shall be not less than 1:5000.

3.1.2 Test for murine viruses

Carry out the test for murine virus detection (Appendix VIII H). No specified murine viruses shall be detected.

3.1.3 Test for mycoplasmas

It complies with the test for mycoplasmas (Appendix VIII B).

3.2 Control tests on bulk

3.2.1 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.2.2 pH

The pH shall be 6.5-7.5 (Appendix V A).

3.2.3 Isoelectric point

The isoelectric point of the preparation shall be the same as that of the reference substance (Appendix IV D).

3.2.4 Purity

3.2.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). The concentration of separating gel is 10%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. If non-reducing SDS-PAGE is used, the results shall be the same as that of reference substance. If reducing SDS-PAGE is used, the heavy and light chains of immunoglobulin shall be not less than 95.0% based on densitometer scanning.

3.2.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method with the gel for separating 10-500 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer with pH 7.0. The amount of loading sample shall be not less than 20 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 based on the peak of absorption. The area of major immunoglobulin peak shall be not less than 95.0% of the total area.

3.2.5 Residual murine myeloma DNA

The residual murine myeloma DNA shall be not more than 100 pg/dose (Appendix IX B).

3.3 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix VIII A).

3.4 Control tests on final product

Other than the tests for moisture content and reconstitution time, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.4.1 Identity test

Reactivity with normal human peripheral blood mononuclear cells shall be tested using immunofluorescent method. The reference value in normal range shall be $66.0\% \pm 10\%$.

3.4.2 Physical inspection

3.4.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid after reconstitution and opalescence may occur.

3.4.2.2 Reconstitution time

The preparation shall be reconstituted completely within 5 minutes after adding 1 ml of physiological saline.

3.4.2.3 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.4.2.4 Weight variation

It complies with the test for weight variation (Appendix I A). The quantity shall be not less than the stated value.

3.4.3 Chemical tests**3.4.3.1 Moisture content**

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.4.3.2 pH

The pH shall be 6.5-7.5 (Appendix V A).

3.4.3.3 Protein content

It shall be 90%-110% of the labelled concentration (Appendix VI B, method 2).

3.4.4 Titer determination

Reactivity with normal human peripheral blood mononuclear cells shall be tested using immunofluorescent method. The reference value in normal range shall be $66.0\% \pm 10\%$ and the antibody titer shall be not less than $1 : 10000$.

3.4.5 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.4.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

3.4.7 Pyrogen test

It complies with the test for pyrogen (Appendix XIII D). The injecting dose of the product shall be 2 mg/kg of rabbit body weight.

4 Storage, shipping and validity period

Store and ship at 2-8°C. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Botulinum Toxin Type A for Injection

Botulinum toxin type A (BTA) for injection is a freeze-dried preparation made from crystalline BTA with a stabilizer.

1 Basic requirements

The facilities, water, source and subsidiary materials, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Manufacturing**2.1 Bacterial seeds**

The Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics shall apply.

2.1.1 Name and origin of bacterial strains

The strains of *Clostridium botulinum* type A (Hall strain or the other approved strains) used for production shall be highly toxin producing.

2.1.2 Establishment of seed lot

The Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics shall apply.

2.1.3 Passage of seed lot

The seed from master shall be subcultured for not more than five passages and that from working seed lots shall be subcultured for not more than ten passages.

2.1.4 Control tests on seed lots

Semisolid media of gelatin and agar, blood agar plate, lactose yolk agar plate or other appropriate media shall be used for control tests. The bacterial seeds shall be of typical morphology, and cultural and biochemical characteristics. It shall produce BTA of not less than 1.0×10^5 LD₅₀/ml.

2.1.5 Storage of seed lots

Master and working seed lots shall be lyophilized and stored at 2-8°C. The working seed lots may also be preserved at -20°C in semisolid medium of gelatin agar.

2.2 Crystalline toxin**2.2.1 Crude toxin****2.2.1.1 Medium for toxin producing**

Media composed of trypticase, yeast dialysate (or yeast extract) and glucose or other approved media can be used.

2.2.1.2 Inoculate the bacterial seed into the toxin producing medium and incubate at a suitable temperature for a certain time.

2.2.1.3 At the end of incubation, take samples from each container for bacterial purity test by microscopic examination. No contaminating microorganisms shall be found, and then the cultures shall be sterilized by filtration. The yield is regarded as crude toxin and its toxicity shall be 10^5 - 10^6 LD₅₀/ml.

2.2.2 Purification and crystallization of toxin

2.2.2.1 The crude toxin can be purified by the procedures including isoelectric precipitation, RNase treatment, ion-exchange chromatography, ammonium sulfate concentration, dialysis and

natural crystallization, or other approved method.

2.2.2.2 In the course of crystallization, an appropriate preservative can be added into external liquid for dialysis.

2.2.3 Storage of crystalline toxin

After crystallization, the purified toxin shall be stored at 2-8°C and protected from light.

2.2.4 Control tests on crystalline toxin

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

2.3.1.1 The toxin shall be dissolved and dialyzed with PB, and tested for toxicity after sterilization by filtration.

2.3.1.2 The filtrated toxin of known toxicity shall be diluted with physiological saline to a suitable concentration.

2.3.1.3 Add a certain volume of diluted toxin to a quantity of stabilizer solution. The toxicity (LD_{50}/ml) of the final bulk shall be within the specified range.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. The temperature of the preparation during lyophilization shall be not higher than 35°C. The final containers shall be sealed under vacuum or after filling with nitrogen.

2.4.3 Specifications

50-150 U of BTA per container.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on crystalline toxin

3.1.1 Crystalline toxin shall be homogenous, needle-shaped crystals under a high power microscope.

3.1.2 Toxicity test

Any of the following methods shall be selected to perform the test for toxicity. The toxicity of crystalline toxin shall be 1.0×10^5 - 1.0×10^6 LD_{50}/ml .

3.1.2.1 After being dissolved and dialyzed, the crystalline toxin shall be properly diluted. Determine its toxicity by Broff method, and calculate the potency of crystalline toxin. Inoculate i. v. 0.1 ml of the toxin (or the diluted

toxin) into each of five mice weighing 14-16 g. Observe the mice for a period and record the average survival time (min). A standard dose-response curve (amount of toxin vs survival time) can be plotted, thereby the toxicity of the toxin (or the diluted toxin) can be calculated.

3.1.2.2 Serially dilute the toxin. Inject 0.5 ml of each dilution i. p. into each of four mice weighing 14-16 g. Record the number of death within 4 days. Calculate the toxicity (LD_{50}) of the toxin (or the diluted sample) statistically (Reed-Muench method).

3.1.3 Purity

3.1.3.1 Based on the protein concentration determined by spectrophotometer at 280 nm and the toxicity of the crystalline toxin (Section 3.1.2), the purity of the crystalline toxin can be calculated. It shall be more than 1.0×10^7 LD_{50}/mg Pr.

3.1.3.2 The ratio of A_{260} to A_{280} for crystalline toxin shall be not more than 0.6.

3.1.4 Type specificity of toxin

Reconstitute the freeze-dried diagnostic sera (types A, B, C, D, E and F) in each container with 1 ml of water for injection. Add 1 ml of toxin being tested containing about 100 LD_{50} into each container of reconstituted diagnostic serum. Meanwhile use another two tubes each containing 1 ml of toxin solution plus 1 ml of physiological saline as controls. Boil one of the tubes for 20 minutes as a negative toxin control, and the other tube is used as a positive toxin control. Incubate the toxin control tubes and the containers containing toxin and serum at 37°C for 45 minutes for neutralization. Inject i. p. 0.5 ml of each mixture separately into each of two to three mice weighing 14-16 g. Observe the animals for 4 days. The toxin being tested shall be botulinum toxin type A.

3.2 Control tests on final bulk

3.2.1 Sterility test

It complies with the test for sterility (Appendix XII A).

3.2.2 Potency test

See Section 3.3.4. It shall be 80%-120% of the stated value.

3.3 Control tests on final product

Other than the determination of residual moisture, sterile physiological saline shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

See Section 3.1.4. At least one container from each final batch shall be sampled for identity test.

3.3.2 Inspection on final containers

The preparation looks like a white crisp cake and turns into a clear, colourless or yellowish liquid



after reconstitution with 1 ml of physiological saline and shaking gently.

3.3.3 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.4 Potency test

Ten to twenty containers of the final product from each batch shall be sampled for potency test. Add 1 ml of physiological saline to each container. Mix the equal volume of the reconstituted toxin and make serial dilutions. Inject i. p. 0.5 ml of each dilution into each of four mice weighing 14-16 g. Observe the mice for 4 days and calculate the LD₅₀ per container statistically. The LD₅₀ per container (1 LD₅₀ is defined as 1 unit) shall be within 80%-120% of the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix VII A).

3.3.6 Diluent

The diluent for reconstitution of final product is sodium chloride injection.

4 Storage, shipping and validity period

Store and ship at -5 to -20°C, protected from light. The validity period is 36 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 1b$ for Injection

Recombinant human interferon $\alpha 1b$ (rhIFN $\alpha 1b$) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interferon $\alpha 1b$ gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN $\alpha 1b$ engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon $\alpha 1b$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 1b$ in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 1b$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be

monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be used. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^7 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method: Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g.

Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.4 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content shall be not more than 100 ng/dose (Appendix IX L).

3.1.8 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.9 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.10 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/300000 IU (Appendix XII E, the limit test of gel-clot method).

3.1.11 Isoelectric point

The isoelectric point of rhIFN α 1b protein shall be pH 4.0-6.5 (Appendix IV D).

3.1.12 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon α 1b reference substance (Appendix VII E).

3.1.14 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Asp-Asn-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/300000 IU (Appendix VIII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted preparation shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution, free of visible insolubles.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less

than 10 EU/vial (Appendix VIII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F, mouse method).

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon α 1b Injection

Recombinant human interferon α 1b (rhIFN α 1b) injection is a liquid preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interferon α 1b gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered cell line

rhIFN α 1b engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon α 1b gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working

seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN α 1b in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon α 1b reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be used. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^7 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method: Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0.

The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm.



The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.4 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content of murine IgG shall be not more than 100 ng/dose (Appendix IX L).

3.1.8 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.9 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.10 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/300000 IU (Appendix XIII E, the limit test of gel-clot method).

3.1.11 Isoelectric point

The isoelectric point of rhIFN α 1b protein shall be pH 4.0-6.5 (Appendix IV D).

3.1.12 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon α 1b reference substance (Appendix VIII E).

3.1.14 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Asp-Asn-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/300000 IU (Appendix XIII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear liquid.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XIII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F, mouse method).

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon α 1b Eye Drops

Recombinant human interferon α 1b (rhIFN α 1b) eye drops is a preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interferon α 1b gene. The recombinant proteins are isolated, purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production

and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN α 1b engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon α 1b gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN α 1b in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon α 1b reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation.

The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Purification

The purification shall be performed using the approved methods. The purity shall meet the requirements in Section 3.1. The preparation of bulk interferon shall be stored at an appropriate temperature after addition of a suitable stabilizer. The storage period for bulk shall be defined.

2.2.6 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 8.0×10^6 IU/mg of protein.

3.1.4 Purity

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 80.0%, and amounts of other proteins with molecular weights of more than 50 kD shall be not more than 10% based on densitometer scanning.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.4 \text{ kD} \pm 10\%$.

3.1.6 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content of murine IgG shall be not more than 100 ng/dose (Appendix IX L).

3.2 Control tests on final bulk

3.2.1 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a liquid in light yellow colour.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the test for minimum fill (Appendix V F). The quantity shall be not less than the stated value.

3.3.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value

(Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2a$ for Injection

Recombinant human interferon $\alpha 2a$ (rhIFN $\alpha 2a$) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interferon $\alpha 2a$ gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN $\alpha 2a$ engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon $\alpha 2a$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 2a$ in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2a$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method: Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm. The number of theoretical



plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μg . Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content of murine IgG shall be not more than 100 ng/dose (Appendix IX L).

3.1.8 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.9 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.10 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.1.11 Isoelectric point

The isoelectric point of rhIFN $\alpha 2a$ protein shall be pH 5.5-6.8 (Appendix IV D).

3.1.12 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2a$ reference substance (Appendix VIII E).

3.1.14 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix

XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

It shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2a$ Injection

Recombinant human interferon $\alpha 2a$ (rhIFN $\alpha 2a$) injection is a liquid preparation prepared from recombinant proteins expressed by *E. coli*

containing recombinant plasmids of the human interferon $\alpha 2a$ gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN $\alpha 2a$ engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon $\alpha 2a$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 2a$ in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2a$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of

Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method: Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content of murine IgG shall be not more than 100 ng/dose (Appendix IX L).

3.1.8 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.9 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.10 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.1.11 Isoelectric point

The isoelectric point of rhIFN $\alpha 2a$ protein shall be pH 5.5-6.8 (Appendix IV D).

3.1.12 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2a$ reference substance (Appendix VIII E).

3.1.14 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear liquid.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 Chemical tests

3.3.3.1 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.3.2 Test for residual polysorbate 80 content

Carry out the test for residual polysorbate 80 (Appendix VI H). If product contains polysorbate 80, the content shall be 0.008%-0.02%.

3.3.3.3 Residual content of methylparaben and propylparaben

If preparation contains methylparaben, the content shall be 0.04%-0.1%. If preparation contains propylparaben, the content shall be 0.004%-0.01%.

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2a$ for Injection (Yeast)

Recombinant human interferon $\alpha 2a$ (rhIFN $\alpha 2a$) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by yeast containing recombinant plasmids of the human interferon $\alpha 2a$ gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed yeast. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered yeast strain
rhIFN $\alpha 2a$ engineered strain is a yeast strain transformed with plasmids containing the human interferon $\alpha 2a$ gene.

2.1.2 Establishment of seed lot system

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on yeast seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on SD agar plates

All colonies that grow on the plates shall have typical morphology of yeast colonies with no evidence of contamination.

2.1.3.2 Morphology of yeast

The test sample examined under light microscope shall show the typical morphology of yeast.

2.1.3.3 Expression level of rhIFN

The expression level of rhIFN $\alpha 2a$ in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.4 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2a$ reference serum.

2.1.3.5 Stability of interferon gene

At least 50 colonies on SD agar plates shall be tested for interferon gene by PCR. The positive rate shall be not lower than 95%.

2.2 Preparation of bulk

2.2.1 Preparation of seed

The yeast of working seed lot qualified in control tests shall be cultured in an appropriate medium as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation.

2.2.4 Processing fermentation products

The yeast mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved methods. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method; Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample

shall be not less than 20 μ g. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of major peak of absorption shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host yeast proteins

It shall be not more than 0.050% of total proteins (Appendix IX E).

3.1.8 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XIII E, the limit test of gel-clot method).

3.1.9 Isoelectric point

The isoelectric point of rhIFN $\alpha 2a$ protein shall be pH 5.7-6.7 (Appendix IV D).

3.1.10 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.11 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2a$ reference substance (Appendix VIII E).

3.1.12 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XIII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XIII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2a$ Vaginal Suppository

Recombinant human interferon $\alpha 2a$ (rhIFN $\alpha 2a$) vaginal suppository is a preparation made by adding rhIFN $\alpha 2a$ to the suppository bases, then molding and film-coating.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial

strain

rhIFN $\alpha 2a$ engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon $\alpha 2a$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 2a$ in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2a$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the

temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Preparation of the suppository

2.3.1 Formulation and sterilization by filtration

The approved formula shall be used. The suppository bases shall be non-irritating to the vaginal canal. After insertion, the suppository shall be softened, melted and dissolved, and mixed with vaginal secretions, and the interferon shall be released gradually to develop the local or systemic effects.

2.3.2 Temperature during mixing interferon with bases shall be not higher than 56°C. Interferon and bases shall be mixed well.

2.3.3 Molding

The approved production process shall be adopted. The suppository shall be intact and smooth. Certain hardness is required to prevent from disfiguring during packaging and storage.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Specifications

The approved specification (s) shall apply.

2.4.3 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method; Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content of murine IgG shall be not more than 100 ng/dose (Appendix IX L).

3.1.8 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.9 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.10 Isoelectric point

The isoelectric point of rhIFN $\alpha 2a$ protein shall be pH 5.5-6.8 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.12 Peptide mapping (to be tested at least

once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2a$ reference substance (Appendix VIII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final product

3.2.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.2.2 Physical inspection

3.2.2.1 Appearance

The suppository shall be white or yellowish in colour, externally even, smooth and hard.

3.2.2.2 Weight deviation

It complies with the weight deviation test (Appendix I B).

3.2.2.3 Disintegration test for suppositories

It complies with the disintegration test for suppositories (Appendix V D).

3.2.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.2.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.2.5 Microbial limit test

It complies with the microbial limit test (Appendix XII G).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of molding.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2b$ for Injection

Recombinant human interferon $\alpha 2b$ (rhIFN $\alpha 2b$) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interferon $\alpha 2b$ gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production

and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain
rhIFN $\alpha 2b$ engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon $\alpha 2b$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 2b$ in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2b$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method; Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm. The limit test of gel-clot method shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content of murine IgG shall be not more than 100 ng/dose (Appendix IX L).

3.1.8 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.9 Activity of residual antibiotics

It complies with test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.10 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix VIII E, the limit test of gel-clot method).

3.1.11 Isoelectric point

The isoelectric point of rhIFN $\alpha 2b$ protein shall be pH 4.0-6.7 (Appendix IV D).

3.1.12 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2b$ reference substance (Appendix VIII E).

3.1.14 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix VIII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

It shall be not more than 3.0%. If preparation contains glucose, the residual moisture content shall be not more than 4.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix VIII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light.

The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2b$ Injection

Recombinant human interferon $\alpha 2b$ (rhIFN $\alpha 2b$) injection is a liquid preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interferon $\alpha 2b$ gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN $\alpha 2b$ engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon $\alpha 2b$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no

evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 2b$ in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2b$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the

requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. Validity period shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method; Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual murine IgG

The content of residual murine IgG shall be tested if affinity chromatography with mouse monoclonal antibody is used in purification. The content of murine IgG shall be not more than 100 ng/dose (Appendix IX L).

3.1.8 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.9 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.10 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.1.11 Isoelectric point

The isoelectric point of rhIFN $\alpha 2b$ protein shall be pH 4.0-6.7 (Appendix IV D).

3.1.12 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2b$ reference substance (Appendix VIII E).

3.1.14 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear liquid.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2b$ for Injection (*P. putida*)

Recombinant human interferon $\alpha 2b$ (rhIFN $\alpha 2b$) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by *Pseudomonas putida* containing recombinant

plasmids of the human interferon $\alpha 2b$ gene. The recombinant proteins are isolated and purified after fermentation of the transformed *Pseudomonas putida*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN $\alpha 2b$ engineered bacterial strain is a *Pseudomonas putida* VG-4 strain transformed with plasmids containing the human interferon $\alpha 2b$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *Pseudomonas putida* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be rod-shaped and typically Gram-negative, have capsule but have no spore.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain: Streptomycin sulfate 150 $\mu\text{g/ml}$, Tetracycline hydrochloride 50 $\mu\text{g/ml}$, Ampicillin 100 $\mu\text{g/ml}$.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination of test sample shall reveal typical morphology of *Pseudomonas putida*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *Pseudomonas putida*, which are unable to liquefy gelatin, unable to hydrolyze starch and poly- β -hydroxybutyric acid into monosaccharide (Appendix XIV), unable to utilize denitrification to perform anaerobic respiration, but able to synthesize fluorescein.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 2b$ in cultures on shaker shall be not lower than that of the primary seed lot (1.0×10^9 IU/L).

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2b$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using high-speed centrifugation. The bacterial mass can be stored at -20°C or below for 12 months.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This

preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk
See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method; Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.02% of total proteins (Appendix IX D).

3.1.8 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.9 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XIII E, the limit test of gel-clot method).

3.1.10 Isoelectric point

The isoelectric point of rhIFN $\alpha 2b$ protein shall be pH 5.7-6.7 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy

The maximum absorption peak shall be at $278 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.12 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2b$ reference substance (Appendix VIII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XIII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles

(Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0%. If preparation contains glucose, the residual moisture content shall be not more than 4.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix VII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon $\alpha 2b$ Injection (*P. putida*)

Recombinant human interferon $\alpha 2b$ (rhIFN $\alpha 2b$) injection is a liquid preparation prepared from recombinant proteins expressed by *Pseudomonas putida* containing recombinant plasmids of the human interferon $\alpha 2b$ gene. The recombinant proteins are isolated and purified after fermentation of the transformed *Pseudomonas putida*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN $\alpha 2b$ engineered bacterial strain is a *Pseudomonas putida* VG-4 strain transformed with plasmids containing the human interferon $\alpha 2b$ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *Pseudomonas putida* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be rod-shaped and Gram-negative, have capsule but have no spore.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivities of the bacteria shall be the same as that of the original strain: Streptomycin sulfate 150 $\mu\text{g/ml}$, Tetracycline hydrochloride 50 $\mu\text{g/ml}$, Ampicillin 100 $\mu\text{g/ml}$.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *Pseudomonas putida*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *Pseudomonas putida*, which are unable to liquefy gelatin, unable to hydrolyze starch, starch and poly- β -hydroxybutyric acid into mono-saccharide (Appendix XIV), unable to utilize denitrification to perform anaerobic respiration, but able to synthesize fluorescein.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN $\alpha 2b$ in cultures on shaker shall be not less than that of the primary seed lot (1.0×10^9 IU/L).

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon $\alpha 2b$ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacteria of working seed lot qualified in control tests shall be cultured in an appropriate

medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using high-speed centrifugation. The bacterial mass can be stored at -20°C or below for 12 months.

2.2.5 Preliminary purification

The approved purification process shall be adopted. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at $2-8^{\circ}\text{C}$.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply. It shall be not less than 1.1 ml per vial and 1.04 ml per syringe.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^8 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μg with Silver stain or not less than 10 μg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method; Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μg . Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μg . Molecular weight of the sample shall be $19.2 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.02% of total proteins (Appendix IX D).

3.1.8 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other

antibiotics.

3.1.9 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.1.10 Isoelectric point

The isoelectric point of rhIFN $\alpha 2b$ protein shall be pH 5.7-6.7 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy

The maximum absorption peak shall be at 278 nm \pm 3 nm (Appendix II A).

3.1.12 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon $\alpha 2b$ reference substance (Appendix VIII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser-Arg-Arg-Thr-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/3000000 IU (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear liquid.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XII E, the limit test of

gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interferon γ for Injection

Recombinant human interferon γ (rhIFN γ) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interferon γ gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIFN γ engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interferon γ gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIFN

The expression level of rhIFN γ in cultures on shaker shall be not less than that of the primary seed lot.

2.1.3.7 Type of interferon expressed

Type of interferon expressed shall be confirmed by neutralization test using anti-interferon γ reference serum.

2.1.3.8 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be used. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interferon. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interferon with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.5×10^7 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% (including monomer and dimer) based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method: Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g. Wavelength for detection is 280 nm. The number of theoretical

plates of column shall be not less than 1000 calculated based on the peak of absorption. The area of absorption of major interferon peak (including monomer and dimer) shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the sample shall be 16.8 kD±10%.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.8 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.9 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/1000000 IU (Appendix XII E, the limit test of gel-clot method).

3.1.10 Isoelectric point

The isoelectric point of rhIFN γ protein shall be pH 8.1-9.1 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy

The maximum absorption peak shall be at 278 nm±3 nm (Appendix II A).

3.1.12 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the interferon γ reference substance (Appendix VIII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Gln-Asn-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/1000000 IU (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall

be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X C).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Interleukin-2 for Injection

Recombinant human interleukin-2 (rhIL-2) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human interleukin-2 gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation

contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhIL-2 engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the human interleukin-2 gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhIL-2

The expression level of rhIL-2 in cultures on shaker shall be not less than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation.

The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be used. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk interleukin-2. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk interleukin-2 with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X D).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^7 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 μ g with Silver stain or not less than 10 μ g with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method; Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 μ g.

Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1500 calculated based on the peak of absorption. The area of absorption of major interleukin-2 peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 μ g. Molecular weight of the sample shall be $15.5 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.8 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics. If preparation contains SDS, the concentration of SDS must be diluted to less than 0.01% for this test.

3.1.9 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/1000000 IU (Appendix XII E, limit test of gel-clot method). If preparation contains SDS, the concentration of SDS must be diluted to less than 0.0025% for this test.

3.1.10 Isoelectric point

The isoelectric point of rhIL-2 protein shall be pH 6.5-7.5 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy

The maximum absorption peak shall be at $277 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.12 Peptide mapping (to be tested at least once a year)

The profile of peptide map shall be in consistency with that of the interleukin-2 reference substance (Appendix VIII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Ala-Pro-Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/1000000 IU (Appendix XII E, limit test of gel-clot method). If preparation contains SDS, the concentration of SDS must be diluted to less than 0.0025% for this test.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5. If the product does not contain SDS, it shall be 3.5-7.0 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X D).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XIII E, limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F, mouse method).

3.3.8 Content of residual acetonitrile

If acetonitrile is involved in the process, content of residual acetonitrile shall be tested using gas chromatography (Appendix III C). Chromatography column: Quartz capillary column, column temperature: 45°C, vaporizer temperature: 150°C, detector temperature: 300°C, carrier gas: nitrogen, flow speed: 4.0 ml/min. Prepare 4.0 mg/L standard acetonitrile solutions with water. One ml of the testing samples and each standard acetonitrile solution are injected into the vials and heat calibrated for 30 minutes in a micro-sample processing device at 80°C. A portion of 400 µl of each sample is loaded. Linear regression analysis is carried out to obtain content of residual acetonitrile in the testing samples. The content of residual acetonitrile in the preparation shall be not more than 0.0004%.

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Erythropoietin for Injection (CHO Cell)

Recombinant human erythropoietin (rhEPO) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by Chinese hamster ovarian (CHO) cells containing recombinant plasmids of the human erythropoietin gene. The recombinant proteins are isolated, purified and lyophilized after cultivations of the transfected CHO cells. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered cell line

2.1.1 Name and origin of engineered cell line

rhEPO engineered cell line is a Chinese hamster ovarian cell line with the dihydrofolate reductase gene defective (CHO-dhfr) transformed with plasmids containing the human erythropoietin gene.

2.1.2 Establishment, passage and storage of cell banks

The master cell bank was established by proliferation of cells from the primary cell bank. The working cell bank is established by proliferation of cells from the master cell bank. The number of passages shall be not more than the approved limit. The cell lines are stored in liquid nitrogen. The cell lines shall be qualified in control tests before being used for production.

2.1.3 Control tests on master and working cell banks

The Requirements for Preparations and Control Tests of Animal Cells as Substrates Used for Production of Biologics shall apply.

2.1.3.1 Tests for extraneous agents

Cells shall be free of bacteria and fungi, mycoplasmas and viruses.

2.1.3.2 Identity test for cells

Biochemical tests such as isoenzyme analysis, immunological tests, cytological tests and genetic markers tests shall be used. All test results shall meet the criteria of a typical CHO cell.

2.1.3.3 Expression level of rhEPO

The expression level of rhEPO in the cells from working cell bank shall be not lower than that in the cells from primary cell bank.

2.2 Bulk

2.2.1 Recovery and proliferation of cells

Cells from the working cell bank are recovered, cultured and proliferated in the medium containing inactivated calf serum. A quantity of cells is prepared for cultivations in rolling bottle or bioreactor. The quality of calf serum shall comply with the relevant requirements in Appendix XIII D.

2.2.2 Culture medium

Cell culture medium used for production must be free of calf serum and antibiotics.

2.2.3 Cultivation of cells

Sterile procedure shall be applied in the process of cultivation of cells. The period of cultivation depends on the growth rate of cells.

2.2.4 Isolation and purification

The approved procedures for isolation and purification



of rhEPO from the supernatant of cell cultures shall be used. The bulk rhEPO are prepared through processes of the ultrafiltration concentration and the multi-step chromatographic purification. rhEPO shall be stored in an appropriate temperature after sterilization by filtration. The storage period for bulk shall be defined.

2.2.5 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

Final bulk is prepared by adding appropriate stabilizer into bulk, then diluted with buffer solution and sterilized by filtration.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply. Filling and lyophilization shall be conducted immediately after sterilization by filtration. The temperature of the preparation during lyophilization shall be not higher than 30°C.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.2 Biological activity test

3.1.2.1 Activity test in vivo

Carry out the test in vivo for biological activity (Appendix X B).

3.1.2.2 Activity test in vitro

Carry out the test in vitro for biological activity according to the instructions of ELISA diagnostic kit.

3.1.3 Specific activity

The specific activity shall be not less than 1.2×10^5 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). Non-reducing SDS-PAGE and Coomassie brilliant blue stain shall be used. The concentration of separating gel is 12.5%. The amount of loading sample shall be not less than

10 µg. Purity shall be not less than 98.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). HPLC-SEC method; Chromatography column is hydrophilic silicon gel size exclusion chromatographic column, exclusion limit is 300 kD, granularity is 10 µm, porosity is 24 nm, internal diameter is 7.5 mm and column length is 30 cm. Flow phase is 3.2 mmol/L disodium hydrogen phosphate-1.5 mmol/L of potassium dihydrogen phosphate-400.4 mmol/L sodium chloride, pH 7.3. Amount of loading sample is 20-100 µg. Detection wavelength is 280 nm. The number of theoretical plates of column shall be not less than 1500 calculated based on the peak of absorption. The purity shall be not less than 98.0% calculated based on the area normalization method.

3.1.5 Molecular weight

Carry out the test for purity by electrophoresis (Appendix IV C). Reducing SDS-PAGE and Coomassie brilliant blue R-250 stain are used. The concentration of separating gel is 12.5%. The sample loaded shall be not less than 1 µg. Molecular weight shall be 36-45 kD.

3.1.6 UV spectrometry

Maximum absorption peak shall be at $279 \text{ nm} \pm 2 \text{ nm}$, and minimum absorption peak shall be at $250 \text{ nm} \pm 2 \text{ nm}$. There shall be no absorption at 320-360 nm (Appendix II A).

3.1.7 Isoelectric point

The isoelectric point of rhEPO shall be pH 3.3-4.3 using ampholyte buffer of pH 3.0-5.0 (Appendix IV D).

3.1.8 Content of sialic acid

The content of sialic acid shall be not less than 9.0 mol/mol rhEPO (Appendix VI C).

3.1.9 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 100 pg/ 10^4 IU rhEPO (Appendix IX B).

3.1.10 Content of residual host CHO cell proteins

The content of residual CHO cell proteins shall be not more than 0.10% of the total protein content determined by immunoenzymometric assay.

3.1.11 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 2 EU/ 10^4 IU rhEPO (Appendix XIII E, the limit test of gel-clot method).

3.1.12 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 0.01% determined by ELISA.

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rhEPO reference substance using

reverse phase HPLC after digestion with trypsin (Appendix VIII E). Samples are dialyzed and lyophilized. The samples are reconstituted to a concentration of 1.5 mg/ml with 1% ammonium bicarbonate solution. Add trypsin into the samples and incubate at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 6 hours. Chromatography column is reverse phase C_8 column (25 cm \times 4.6 mm ID, granularity 5 μm , porosity 30 nm). Column temperature is $45^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, flow rate is 0.75 ml/min and loading volume of sample is 20 μl . Gradient elution conditions are followed as the table below (A: 0.1% trifluoroacetic acid in water, B: 0.1% trifluoroacetic acid in 80% acetonitrile solution).

No.	Time (min)	Flow rate (ml/min)	A(%)	B(%)
1	0.00	0.75	100.0	0.0
2	30.00	0.75	85.0	15.0
3	75.00	0.75	65.0	35.0
4	115.00	0.75	15.0	85.0
5	120.00	0.75	0.0	100.0
6	125.00	0.75	100.0	0
7	145.00	0.75	100.0	0

3.1.14 N-terminal amino acid sequence (to be determined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than $2 \text{ EU}/10^3 \text{ IU rhEPO}$ (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the tests for reconstitution time and moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Reconstitution time

The time for reconstitution shall be not more than 2 minutes after adding the stated amount of sterile

water for injection.

3.3.2.3 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.4 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

The pH shall be 6.4-7.4 or 5.4-6.4 (Appendix V A).

3.3.3.3 Content of sodium

The content of sodium shall be not more than 190 mmol/L (Appendix VII J).

3.3.3.4 Content of citrate

The content of citrate shall be not more than 25 mmol/L (Appendix VII H, method 2).

3.3.3.5 Protein content

Carry out the test for protein content (Appendix VI B, method 2). The content shall meet the requirements.

3.3.4 Biological activity tests

3.3.4.1 Activity test in vitro

Carry out the test in vitro for biological activity according to the instructions of ELISA diagnostic kit. The activity in vitro shall be 80%-120% of the stated value (Appendix X B).

3.3.4.2 Activity test in vivo

Carry out the test in vivo for biological activity (Appendix X B). The activity in vivo shall be 80%-140% of the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than $2 \text{ EU}/10^3 \text{ IU rhEPO}$ (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at $2-8^{\circ}\text{C}$, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Erythropoietin Injection (CHO Cell)

Recombinant human erythropoietin (rhEPO) injection is a liquid preparation prepared from recombinant proteins expressed by Chinese hamster ovarian (CHO) cells containing recombinant plasmids of the human erythropoietin gene. The recombinant proteins are isolated and purified after cultivations of the transfected CHO cells. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered cell line

2.1.1 Name and origin of engineered cell line
rhEPO engineered cell line is a Chinese hamster ovarian cell line with the dihydrofolate reductase gene defective (CHO-dhfr⁻) transformed with plasmids containing the human erythropoietin gene.

2.1.2 Establishment, passage and storage of cell banks

The master cell bank was established by proliferation of cells from the primary cell bank. The working cell bank is established by proliferation of cells from the master cell bank. The number of passages shall be not more than the approved limit. The cell lines are stored in liquid nitrogen. The cell lines shall be qualified in control tests before being used for production.

2.1.3 Control tests for master and working cell banks

It shall comply with Requirements for Preparations and Control Tests of Animal Cells as Substrates Used for Production of Biologics.

2.1.3.1 Tests for extraneous agents

Cells shall be free of bacteria and fungi, mycoplasmas and viruses (Appendix XIII C).

2.1.3.2 Identity test on cells

Biochemical tests such as isoenzyme analysis, immunological tests, cytological tests and genetic markers tests shall be used. All test results shall meet the criteria of a typical CHO cell.

2.1.3.3 Expression level of rhEPO

The expression level of rhEPO in the cells from working cell bank shall be not lower than that in the cells from primary cell bank.

2.2 Bulk

2.2.1 Recovery and proliferation of cells

Cells from the working cell bank are recovered, cultured and proliferated in the medium containing inactivated calf serum. A quantity of cells is prepared for cultivations in rolling bottle or bioreactor. The quality of calf serum shall comply with the relevant requirements in Appendix XIII D.

2.2.2 Culture medium

Cell culture medium used for production must be free of calf serum and antibiotics.

2.2.3 Cultivation of cells

Sterile procedure shall be applied in the process of cultivation of cells. The period of cultivation depends on the growth of cells.

2.2.4 Isolation and purification

The approved procedures for isolation and purification of rhEPO from the supernatant of cell cultures shall be used. The bulk rhEPO are prepared through processes of the ultrafiltration concentration and the multi-step chromatographic purification. rhEPO shall be stored in an appropriate temperature after sterilization by filtration. The storage period for bulk shall be defined.

2.2.5 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration
Final bulk is prepared by adding appropriate stabilizer into bulk, then diluted with buffer solution and sterilized by filtration.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.2 Biological activity test

3.1.2.1 Activity tests in vivo

Carry out the test in vivo for biological activity (Appendix X B).

3.1.2.2 Activity tests in vitro

Carry out the test in vitro for biological activity according to the instructions of ELISA diagnostic kit.

3.1.3 Specific activity

The specific activity shall be not less than 1.2×10^5 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). Non-reducing SDS-PAGE and Coomassie brilliant blue stain shall be used. The concentration of separating gel is 12.5%. The amount of loading sample shall be not less than 10 μ g. Purity shall be not less than 98.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). HPLC-SEC method; Chromatography column is hydrophilic silicon gel size exclusion chromatographic column, exclusion limit is 300 kD, granularity is 10 μ m, porosity is 24 nm, internal diameter is 7.5 mm and column length is 30 cm. Flow phase is 3.2 mmol/L disodium hydrogen phosphate, 1.5 mmol/L potassium dihydrogen phosphate and 400.4 mmol/L of sodium chloride, pH 7.3. Amount of loading sample is 20-100 μ g. Detection wavelength is 280 nm. The number of theoretical plates of column shall be not less than 1500 calculated based on the peak of absorption. The purity shall be not less than 98.0% calculated based on the area normalization method.

3.1.5 Molecular weight

Carry out the test for purity by electrophoresis (Appendix IV C). Reducing SDS-PAGE and Coomassie brilliant blue R-250 stain are used. The concentration of separating gel is 12.5%. The sample loaded shall be not less than 1 μ g. Molecular weight shall be 36-45 kD.

3.1.6 UV spectrometry

Maximum absorption peak shall be at $279 \text{ nm} \pm 2 \text{ nm}$, and minimum absorption peak shall be at $250 \text{ nm} \pm 2 \text{ nm}$. There shall be no absorption at 320-360 nm (Appendix II A).

3.1.7 Isoelectric point

The isoelectric point of rhEPO shall be pH 3.3-4.3 using ampholyte buffer of pH 3.0-5.0 (Appendix IV D).

3.1.8 Content of sialic acid

The content of sialic acid shall be not less than 9.0 mol/mol rhEPO (Appendix VI C).

3.1.9 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 100 pg/ 10^4 IU rhEPO (Appendix IX B).

3.1.10 Content of residual CHO cell proteins

The content of residual CHO cell proteins shall be

not more than 0.10% of the total protein content determined by immunoenzymometric assay.

3.1.11 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 2 EU/ 10^4 IU rhEPO (Appendix XII E, the limit test of gel-clot method).

3.1.12 Content of residual bovine serum albumin

The content of residual bovine serum albumin shall be not more than 0.01% determined by ELISA.

3.1.13 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rhEPO reference substance using reverse phase HPLC after digestion with trypsin (Appendix VIII E). Samples are dialyzed and lyophilized. The samples are reconstituted to a concentration of 1.5 mg/ml with 1% ammonium bicarbonate solution. Trypsin is added into the samples and incubated at $37^\circ\text{C} \pm 0.5^\circ\text{C}$ for 6 hours. Chromatography column is reverse phase C_{18} column (25 cm \times 4.6 mm ID, granularity 5 μ m, porosity 30 nm). Column temperature is $45^\circ\text{C} \pm 0.5^\circ\text{C}$, flow rate is 0.75 ml/min and loading volume of sample is 20 μ l. Elution conditions are followed as the table below (A: 0.1% trifluoroacetic acid in water, B: 0.1% trifluoroacetic acid in 80% acetyl-nitrile solution)

No.	Time (min)	Flow rate (ml/min)	A(%)	B(%)
1	0.00	0.75	100.0	0.0
2	30.00	0.75	85.0	15.0
3	75.00	0.75	65.0	35.0
4	115.00	0.75	15.0	85.0
5	120.00	0.75	0.0	100.0
6	125.00	0.75	100.0	0
7	145.00	0.75	100.0	0

3.1.14 N-terminal amino acid sequence (to be determined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 2 EU/ 10^3 IU rhEPO (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.



3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, colourless liquid.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.4-7.4 or 5.4-6.4 (Appendix V A).

3.3.3.2 Content of sodium

The content of sodium shall be not more than 190 mmol/L (Appendix VII J).

3.3.3.3 Content of citrate

The content of citrate shall be not more than 25 mmol/L (Appendix VII H, method 2).

3.3.3.4 Protein content

It complies with the test for protein content (Appendix VI B, method 2). The protein content shall meet the requirements.

3.3.4 Biological activity tests

3.3.4.1 Activity test in vitro

Carry out the test in vitro for biological activity according to the instructions of ELISA diagnostic kit. The activity in vitro shall be 80%-120% of the stated value (Appendix X B).

3.3.4.2 Activity test in vivo

Carry out the test in vivo for biological activity (Appendix X B). The activity in vivo shall be 80%-140% of the stated value.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 2 EU/10³ IU rhEPO (Appendix XIII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with test for abnormal toxicity (Appendix XIII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Granulocyte Colony-stimulating Factor Injection

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) injection is a liquid preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the rhG-CSF gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered cell line

rhG-CSF engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the rhG-CSF gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhG-CSF

The expression level of rhG-CSF in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be used. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk rhG-CSF. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk rhG-CSF with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X E).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 6.0×10^7 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). RP-HPLC method: The matrix of chromatographic column is octadecylsilane bounded silica. Flow phases: phase A (0.1% trichloroacetic acid in water) and phase B (0.1% trichloroacetic acid in acetonitrile solution). Gradient elution (0-70% of phase B) is carried out at room temperature. The amount of loading sample shall be not less than 10 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1500 calculated based on the peak of absorption. The area of absorption of major rhG-CSF peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the sample shall be $18.8 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be

not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host bacterial proteins
The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.8 Activity of residual antibiotics
It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.9 Test for bacterial endotoxin
The content of bacterial endotoxin shall be less than 10 EU/300 µg (Appendix XIII E, the limit test of gel-clot method).

3.1.10 Isoelectric point
The isoelectric point of rhG-CSF protein shall be pH 5.8-6.6 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy
The maximum absorption peak shall be at 278 nm ± 3 nm (Appendix II A).

3.1.12 Peptide mapping (to be tested at least once half a year)
The profile of peptide map shall be in consistency with that of the rhG-CSF reference substance (Appendix VII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)
The N-terminal sequence examined by an amino acid sequencer shall be Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin
The content of bacterial endotoxin shall be less than 10 EU/300 µg (Appendix XIII E, the limit test of gel-clot method).

3.2.2 Sterility test
It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test
Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers
The product shall be a clear liquid.

3.3.2.2 Test for visible particles
It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity
It complies with the requirements for filling quantity (Appendix I A). The quantity shall be not less than the stated value.

3.3.3 pH
It shall be 3.5-4.5 (Appendix V A).

3.3.4 Biological activity
It shall be 80%-150% of the stated value (Appendix X E).

3.3.5 Sterility test
It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for bacterial endotoxin
The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XIII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity
It complies with the test for abnormal toxicity (Appendix XIII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Granulocyte/Macrophage Colony-stimulating Factor for Injection

Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) for injection is a freeze-dried preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the rhGM-CSF gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered cell line
rhGM-CSF engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the rhGM-CSF gene.

2.1.2 Establishment of bacterial seed lots
The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds
The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examinations shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particles or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhGM-CSF

The expression level of rhGM-CSF in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and durations, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be used. The purity shall meet the requirements.

2.2.6 Further purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation,

after further purification, is the bulk rhGM-CSF. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk rhGM-CSF with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification(s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X F).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.0×10^7 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.



3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). SEC-HPLC method: Gel for separating 5-60 kD proteins shall be used. Flow phase is 0.1 mol/L phosphate-0.1 mol/L sodium chloride buffer, pH 7.0. The amount of loading sample shall be not less than 20 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 1500 calculated based on the peak of absorption. The area of absorption of major rhGM-CSF peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the sample shall be 14.5 kD±10%.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.10% of total proteins (Appendix IX C).

3.1.8 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.9 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/300 µg of protein (Appendix XII E, the limit test of gel-clot method).

3.1.10 Isoelectric point

The isoelectric point of rhGM-CSF protein shall be pH 4.7-5.7 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy

The maximum absorption peak shall be at 279 nm±3 nm (Appendix II A).

3.1.12 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rhGM-CSF reference substance (Appendix VIII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be (Met)-Ala-Pro-Ala-Arg-Ser-Pro-Ser-Pro-Ser-Thr-Gln-Pro-Trp-Glu-His.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/300 µg of protein (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination of moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X F).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 10 EU/vial (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Streptokinase for Injection

Recombinant streptokinase for injection is a freeze-

dried preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the streptokinase gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

The recombinant streptokinase engineered bacterial strain is an *E. coli* strain transformed with plasmids containing a streptokinase gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of streptokinase

The expression level of streptokinase in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix X G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Preliminary purification

The approved purification process shall be used. The purity shall meet the requirements.

2.2.6 Final purification

The further purification shall be performed using the approved method. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk streptokinase. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.7 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk streptokinase with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X D).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 9.00×10^4 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 10%. The amount of loading sample shall be not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). RP-HPLC method: The matrix of chromatographic column is octadecylsilane bounded silica. Flow phases: phase A (0.1% trichloroacetic acid in water) and phase B (0.1% trichloroacetic acid in acetonitrile solution). Gradient elution (0-70% of phase B) is carried out at room temperature. The amount of loading sample shall be not less than 10 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 2000 calculated based on the peak of absorption. The area of absorption of major streptokinase peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 10%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the sample shall be $47.0 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Content of residual host bacterial proteins

The content of residual host bacterial proteins shall be not more than 0.050% of total proteins (Appendix IX C).

3.1.8 Activity of residual antibiotics

It complies with the test for residual antibiotics

(Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.1.9 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 3 EU/mg protein (Appendix XII E, the limit test of gel-clot method).

3.1.10 Isoelectric point

The isoelectric point of streptokinase protein shall be pH 4.6-5.6 (Appendix IV D).

3.1.11 Ultraviolet spectroscopy

The maximum absorption peak shall be at $277 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.12 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the streptokinase reference substance (Appendix VIII E).

3.1.13 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Val-Lys-Pro-Val-Gln-Ala-Ile-Ala-Gly-Ser-Glu-Trp-Leu-Leu-Asp.

3.2 Control tests on final bulk

3.2.1 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 3 EU/mg protein (Appendix XII E, the limit test of gel-clot method).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination for moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Weight variation

It complies with the test for weight variation (Appendix I A).

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.9-7.9 (Appendix V A).

3.3.4 Biological activity

It shall be 80%-150% of the stated value (Appendix X D).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3.6 Test for bacterial endotoxin

The content of bacterial endotoxin shall be less than 15 EU/vial (Appendix XII E, the limit test of gel-clot method).

3.3.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XII F, mouse method).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Bovine Basic Fibroblast Growth Factor for External Use, Liquid

Recombinant bovine basic fibroblast growth factor (rbBFGF) is a liquid preparation for external use prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the bovine BFGF gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rbBFGF engineered bacterial strain is an *E. coli* strain transformed with plasmids containing bovine BFGF gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rbBFGF

The expression level of rbBFGF in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.1.3.8 Nucleotide sequence examination

Nucleotide sequence of the bovine BFGF gene shall be the same as the theoretical sequence.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Purification

The approved purification processes shall be used for preliminary and further purifications. The purity shall meet the requirements in Section 3.1.



The preparation, after further purification, is the bulk rbBFGF. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.6 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk rbBFGF with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X G).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.7×10^5 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). RP-HPLC method: The matrix of chromatographic column is octadecylsilane bounded silica. Flow phases; phase A (0.1% trichloroacetic acid in water) and phase B (0.1% trichloroacetic acid in acetonitrile solution). Gradient elution (0-70% of phase B) is carried out at room temperature. The amount of loading sample shall be not less than 10 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 2000 calculated based on the peak of absorption. The area of absorption of major rbBFGF peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the two protein bands of the sample shall be $17.5 \text{ kD} \pm 10\%$ and $22.0 \text{ kD} \pm 10\%$ respectively.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Isoelectric point

The isoelectric point of rbBFGF protein shall be pH 9.0-10.0 (Appendix IV D).

3.1.8 Ultraviolet spectroscopy

The maximum absorption peak shall be at $277 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.9 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rbBFGF reference substance (Appendix VIII E).

3.2 Control tests on final bulk

3.2.1 Biological activity

It complies with the requirement in Appendix X G.

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear liquid, free of visible insoluble particles.

3.3.2.2 Filling quantity

It complies with the test for minimum fill (Appendix V F). The quantity shall be not less than the stated value.

3.3.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 70%-200% of the stated value (Appendix X G).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XI A).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Bovine Basic Fibroblast Growth Factor for External Use

Recombinant bovine basic fibroblast growth factor (rbBFGF) is a freeze-dried preparation for external use prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the bovine BFGF gene. The recombinant proteins are isolated, purified and lyophilized after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rbBFGF engineered bacterial strain is an *E. coli* strain transformed with plasmids containing bovine BFGF gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall

be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rbBFGF

The expression level of rbBFGF in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.1.3.8 Nucleotide sequence examination

Nucleotide sequence of the bovine BFGF gene shall be the same as the theoretical sequence.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

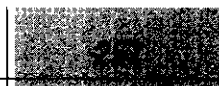
2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Purification

The approved purification processes shall be used for preliminary and further purifications. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk rbBFGF. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.



2.2.6 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk rbBFGF with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X G).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.7×10^5 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). RP-HPLC method: The matrix of chromatographic column is octadecylsilane bounded silica. Flow phases: phase A (0.1% trichloroacetic acid in water) and phase B (0.1% trichloroacetic

acid in acetonitrile solution). Gradient elution (0-70% of phase B) is carried out at room temperature. The amount of loading sample shall be not less than 10 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 2000 calculated based on the peak of absorption. The area of absorption of major rbBFGF peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the two protein bands of the sample shall be $17.5 \text{ kD} \pm 10\%$ and $22.0 \text{ kD} \pm 10\%$ respectively.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Isoelectric point

The isoelectric point of rbBFGF protein shall be pH 9.0-10.0 (Appendix IV D).

3.1.8 Ultraviolet spectroscopy

The maximum absorption peak shall be at $277 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.9 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rbBFGF reference substance (Appendix VIII E).

3.2 Control tests on final bulk

3.2.1 Biological activity

Carry out the test for biological activity (Appendix X G).

3.2.2 Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the tests for reconstitution time and moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution.

3.3.2.2 Reconstitution time

The time for reconstitution shall be not more than 10 minutes after adding the stated amount of sterile water for injection and shaking gently.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 70%-200% of the stated value (Appendix X G).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Bovine Basic Fibroblast Growth Factor Eye Drops

Recombinant bovine basic fibroblast growth factor (rbBFGF) eye drops is a liquid preparation prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the bovine BFGF gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rbBFGF engineered bacterial strain is an *E. coli* strain transformed with plasmids containing bovine BFGF gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no

evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rbBFGF

The expression level of rbBFGF in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.1.3.8 Nucleotide sequence examination

Nucleotide sequence of the bovine BFGF gene shall be the same as the theoretical sequence.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Purification

The approved purification processes shall be used for preliminary and further purifications. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk rbBFGF. The preparation shall be stored at



an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.6 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk rbBFGF with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X C).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 1.7×10^5 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If non-reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix

III B). RP-HPLC method: The matrix of chromatographic column is octadecylsilane bounded silica. Flow phases: phase A (0.1% trichloroacetic acid in water) and phase B (0.1% trichloroacetic acid in acetonitrile solution). Gradient elution (0-70% of phase B) is carried out at room temperature. The amount of loading sample shall be not less than 10 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 2000 calculated based on the peak of absorption. The area of absorption of major rbBFGF peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 15%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the two protein bands of the sample shall be $17.5 \text{ kD} \pm 10\%$ and $22.0 \text{ kD} \pm 10\%$ respectively.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Isoelectric point

The isoelectric point of rbBFGF protein shall be pH 9.0-10.0 (Appendix IV D).

3.1.8 Ultraviolet spectroscopy

The maximum absorption peak shall be at $277 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.9 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rbBFGF reference (Appendix VIII E).

3.2 Control tests on final bulk

3.2.1 Biological activity

Carry out the test for biological activity (Appendix X G).

3.2.2 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a clear, colourless liquid.

3.3.2.2 Test for visible particles

It complies with the test for visible particles (Appendix V B).

3.3.2.3 Filling quantity

It complies with the test for minimum fill (Appendix V F).

3.3.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 70%-200% of the stated value (Appendix X G).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Epidermal Growth Factor for External Use

Recombinant human epidermal growth factor (rhEGF) is a freeze-dried preparation for external use prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human EGF gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhEGF engineered bacterial strain is an *E. coli* strain transformed with plasmids containing human EGF gene.

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhEGF

The expression level of rhEGF in cultures on shaker shall be not lower than that of the primary seed lot.

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods.

2.2.5 Purification

The approved purification processes shall be used for preliminary and further purifications. The purity shall meet the requirements in Section 3.1. The preparation, after further purification, is the bulk rhEGF. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.6 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration



2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk rhEGF with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling and Lyophilization

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X H).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 5.0×10^5 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 17.5%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). RP-HPLC method: The matrix of chromatographic column is octadecylsilane bonded silica. Flow phases: phase A (0.1% trichloroacetic acid in water) and phase B (0.1% trichloroacetic acid in acetonitrile solution). Gradient elution (0-70% of phase B) is carried out at room temperature. The amount of loading sample shall be not less than 10 µg. Wavelength for detection is 280 nm. The number of theoretical plates of

column shall be not less than 500 calculated based on the peak of absorption. The area of absorption of major rhEGF peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 17.5%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the sample shall be $6.0 \text{ kD} \pm 10\%$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Isoelectric point

The isoelectric point of rhEGF protein shall be pH 4.0-5.0 (Appendix IV D).

3.1.8 Ultraviolet spectroscopy

The maximum absorption peak shall be at $275 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.9 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rhEGF reference substance (Appendix VIII E).

3.1.10 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu.

3.1.11 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.1.12 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XII A).

3.3 Control tests on final product

Other than the determination for moisture content, sterile water for injection shall be added as stated on the label, and the reconstituted product shall be subject to the following tests.

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Inspection on final containers

The product looks like a white to off-white crisp cake. It shall change into a clear liquid quickly after reconstitution, free of visible insoluble particles.

3.3.3 Chemical tests

3.3.3.1 Moisture content

The residual moisture content shall be not more than 3.0% (Appendix VII D).

3.3.3.2 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 70%-200% of the stated value (Appendix X H).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Recombinant Human Epidermal Growth Factor Derivative for External Use, Liquid

Recombinant human epidermal growth factor derivative (rhEGF derivative) is a liquid preparation for external use prepared from recombinant proteins expressed by *E. coli* containing recombinant plasmids of the human EGF derivative gene. The recombinant proteins are isolated and purified after fermentation of the transformed *E. coli*. The preparation contains a stabilizer, but free of preservatives and antibiotics.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

2 Production

2.1 Engineered bacterial strain

2.1.1 Name and origin of engineered bacterial strain

rhEGF derivative engineered bacterial strain is an *E. coli* strain transformed with plasmids containing the synthesized human EGF derivative gene [there are three more amino acids (Ala-Arg-Ile) at N-terminal compared with the gene sequence of natural EGF].

2.1.2 Establishment of bacterial seed lots

The Requirements for Preparation and Control of Animal Cell Substrates Used for Production of Biologics shall apply.

2.1.3 Control tests on bacterial seeds

The master seed lot and working seed lot shall be subject to the control tests as follows.

2.1.3.1 Streaking on LB agar plates

All colonies that grow on the plates shall have typical morphology of *E. coli* colonies with no evidence of contamination.

2.1.3.2 Gram-stained smears

The bacteria examined under light microscope shall be typical Gram-negative bacteria.

2.1.3.3 Resistance to antibiotics

The antibiotic sensitivity of the bacteria shall be the same as that of the original strain.

2.1.3.4 Electro-microscopic examination (working seed lot can be exempted)

The examination shall reveal typical morphology of *E. coli*. No contaminations of mycoplasmas, virus-like particle or other microbes shall be observed.

2.1.3.5 Biochemical tests

The bacteria tested shall have biological properties of *E. coli*.

2.1.3.6 Expression level of rhEGF derivative

The expression level of rhEGF derivative in cultures on shaker shall be not lower than that of the primary seed lot (10%).

2.1.3.7 Characterization of plasmid

The map of restriction enzyme digestion shall be the same as that of the original recombinant plasmid.

2.2 Bulk

2.2.1 Preparation of seed

The bacterial seed from working seed lot qualified in control tests shall be cultured in an appropriate medium (a quantity of antibiotic can be used) as an inoculum for fermentation.

2.2.2 Medium for fermentation

A suitable medium shall be used for fermentation. The medium must not contain antibiotics.

2.2.3 Inoculation and fermentation

2.2.3.1 A quantity of seed is inoculated into the sterilized medium.

2.2.3.2 Fermentation conditions, such as the temperature, pH, oxygen dissolving, feeding and duration, shall follow the approved protocol of fermentation for specific production strain. The losing rate of plasmid in bacteria shall be monitored during fermentation at a certain time (Appendix IX G).

2.2.4 Processing fermentation products

The bacterial mass shall be collected and processed using suitable methods. The collected bacteria shall be purified immediately and stored at or below -20°C if necessary. The storage period shall be defined.

2.2.5 Purification

The approved purification process shall be used. The purity shall meet the requirements in Section 3.1. The preparation, after further purification,

is the bulk rhEGF derivative. The preparation shall be stored at an appropriate temperature after addition of a suitable stabilizer and sterilization by filtration. The storage period for bulk shall be defined.

2.2.6 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation and sterilization by filtration

2.3.1.1 Preparation of diluent

The approved formula shall be used. The diluent shall be used immediately after preparation.

2.3.1.2 Dilution and sterilization by filtration

The bulk rhEGF derivative with an appropriate stabilizer and qualified in control tests shall be diluted using the diluent prepared in Section 2.3.1.1 to a desired concentration and sterilized by filtration. This preparation is the final bulk and stored at 2-8°C.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

The approved specification (s) shall apply.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Biological activity

Carry out the test for biological activity (Appendix X H).

3.1.2 Protein content

Carry out the test for protein content (Appendix VI B, method 2).

3.1.3 Specific activity

The ratio of biological activity to protein content shall be not less than 5.0×10^5 IU/mg of protein.

3.1.4 Purity

3.1.4.1 Electrophoresis

Carry out the test for purity by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 17.5%. The amount of loading sample shall be not less than 5 µg with Silver stain or not less than 10 µg with Coomassie brilliant blue R-250 stain. The purity shall be not less than 95.0% based on densitometer scanning.

3.1.4.2 HPLC

Carry out the test for purity by HPLC (Appendix III B). RP-HPLC method: The matrix of chromatographic column is octadecylsilane bounded silica. Flow phases: phase A (0.1% trichloroacetic acid in water) and phase B (0.1% trichloroacetic acid in acetonitrile solution). Gradient elution (0-70% of phase B) is carried out at room temperature. The amount of loading sample shall be not less than 10 µg. Wavelength for detection is 280 nm. The number of theoretical plates of column shall be not less than 500 calculated based on the peak of absorption. The area of absorption of major rhEGF derivative peak shall be not less than 95.0% of the total area.

3.1.5 Molecular weight

Carry out the test for molecular weight by electrophoresis (Appendix IV C). If reducing SDS-PAGE is used, the concentration of separating gel is 17.5%. The amount of loading sample shall be not less than 1.0 µg. Molecular weight of the sample shall be $6.2 \text{ kD} \pm 0.62 \text{ kD}$.

3.1.6 Content of residual extraneous DNA

The content of residual extraneous DNA shall be not more than 10 ng/dose (Appendix IX B).

3.1.7 Isoelectric point

The isoelectric point of rhEGF derivative protein shall be pH 4.1-5.1 (Appendix IV D).

3.1.8 Ultraviolet spectroscopy

The maximum absorption peak shall be at $276 \text{ nm} \pm 3 \text{ nm}$ (Appendix II A).

3.1.9 Peptide mapping (to be tested at least once half a year)

The profile of peptide map shall be in consistency with that of the rhEGF derivative reference substance (Appendix VIII E).

3.1.10 N-terminal amino acid sequence (to be examined at least once a year)

The N-terminal sequence examined by an amino acid sequencer shall be Ala-Arg-Ile-Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly.

3.1.11 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.1.12 Activity of residual antibiotics

It complies with the test for residual antibiotics (Appendix IX A). The preparation must not contain any residual activities of ampicillin or other antibiotics.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test

Immunoblot test (Appendix VIII A) or immunodot test (Appendix VIII B) shall reveal positive results.

3.3.2 Physical inspection

3.3.2.1 Inspection on final containers

The product shall be a colourless and odourless clear liquid, free of visible insoluble particles.

3.3.2.2 Filling quantity

It complies with the test for minimum fill (Appendix V F). The quantity shall be not less than the stated value.

3.3.3 pH

It shall be 6.5-7.5 (Appendix V A).

3.3.4 Biological activity

It shall be 70%-200% of the stated value

(Appendix X H).

3.3.5 Sterility test

It complies with the test for sterility (Appendix XII A).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The approved validity period shall apply, starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.



III Diagnostic Reagents for *in vivo* Test

Purified Protein Derivative of Tuberculin (TB-PPD)

Purified protein derivative of tuberculin (TB-PPD) is a preparation made by cultivation of *Mycobacterium tuberculosis*, sterilization and filtration, followed by purification. It is used for clinical diagnosis of tuberculosis, the selection of eligibles for BCG vaccination and monitoring of the immune response after BCG vaccination.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

The production area for TB-PPD shall comply with the national requirements for an appropriate biosafety level, and shall be separated from the production area for other biologics. All production process of bulk including the inactivation of *Mycobacterium tuberculosis* shall be performed in a completely isolated area. The facilities and apparatus for TB-PPD production shall be used exclusively. The apparatus made of metal or glass or other materials used directly in production shall be washed and cleaned thoroughly and sterilized. The workers engaged in production of TB-PPD shall be healthy. They shall be free from tuberculosis and subjected to one or two X-ray examinations of the chest each year. If suspected tuberculosis is revealed in an X-ray examination, the worker shall be kept away from TB-PPD production temporarily.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The *Mycobacterium tuberculosis* strain CMCC 93009 (H37Rv) shall be used for production of TB-PPD.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and

Quality Control of Biologics.

2.1.3 Passage of seed lots

The total number of passages for a single production harvest from working seed lot shall not exceed 12.

2.1.4 Control tests on seed lots

2.1.4.1 Microscopic examination of stained smears
The bacteria are in short rod or a slightly curved form with round ends by microscopic examination of stained smears. They shall be acid-fast bacilli.

2.1.4.2 Biochemical reactions

The bacilli shall be positive in nitrate reduction test, niacin test and urease test, and negative in catalase test and polysorbate 80 hydrolysis test (Appendix XIV).

2.1.5 Storage of seed lots

The freeze-dried seeds shall be stored at 2-8°C. The liquid seeds shall be stored at or below -70°C.

2.2 Bulk

2.2.1 Working seed for production

The bacterial seed from working seed lot shall be subcultured on Sauton potato medium or modified Sauton synthetic medium. The seed is used for the preparation of bulk.

2.2.2 Production medium

Sauton potato medium, modified Sauton synthetic medium or other approved media shall be used for production.

2.2.3 Inoculation and cultivation

The bacterial seed shall be inoculated onto Sauton potato medium and cultivated at 37°C for 2-3 weeks. One additional passage can be done on Sauton potato medium. The well-grown bacterial pellicles shall be collected from Sauton potato medium and transferred onto the surface of modified Sauton synthetic medium or other suitable media and incubated at 37°C for 1-2 weeks. The well-grown bacterial pellicles shall be selected and transferred onto the surface of modified Sauton synthetic media or other approved media, and incubated at 37°C for 8-10 weeks. The cultures shall be discarded if the submerging, contamination or abnormal growth of pellicle is found during or at the end of cultivation.

2.2.4 Sterilization and filtration

At the end of incubation, the cultures shall be sterilized by autoclaving at 121°C for 30 minutes,

then filtered to remove the pellicle and bacteria.

2.2.5 Collection and storage

The filtrates shall be collected for purification. If the filtrates need storage, 3.0 g/L of phenol or other appropriate preservers shall be added. The filtrates shall be stored at 4-8°C, and the storage period shall not exceed 30 days.

2.2.6 Purification

The protein in the filtrate shall be precipitated by trichloroacetic acid and saturated ammonium sulfate. Purify the precipitates by the approved methods and sterilize by filtration to obtain bulk.

2.2.7 Pooling and lyophilization

2.2.7.1 Pooling

The bulk from not more than five runs of purification shall be pooled.

2.2.7.2 Filling and lyophilization

The bulk qualified in control tests shall be diluted to the specified concentration based on the protein content. The bulk is dispensed into each container, and lyophilized immediately after filling.

2.2.8 Control tests on bulk

See Section 3.1.

2.2.9 Storage and storage period

The bulk shall be stored at 2-8°C. The storage period of the liquid bulk is 60 months starting from the date when the potency test proved qualified. The freeze-dried bulk can be used continually when it is qualified in control tests described in Section 3.1, which shall be carried out every 60 months starting from the date when the potency test proved qualified.

2.3 Final bulk

2.3.1 Formulation

The bulk qualified in control tests shall be diluted to 20 IU/ml or 50 IU/ml with 0.01 mol/L PBS (pH 7.2-7.4, containing 0.0005% polysorbate 80 and 3.0 g/L phenol). The ingredients shall be mixed well during dilution. Samples shall be taken from each bottle for sterility test (Appendix VIII A).

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

1 ml or 2 ml per container. Each single human dose is 0.1 ml containing 5 IU or 2 IU of TB-PPD.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Visual inspection

The freeze-dried bulk looks like a white crisp cake. The reconstituted or liquid bulk shall be a clear, brown liquid, free of insoluble and foreign matters.

3.1.2 Reconstitution time

After adding the water for the injection as stated on the label, the freeze-dried bulk shall be reconstituted completely within 3 minutes.

3.1.3 Moisture content

The moisture content shall be not more than 3.0% (Appendix VII D).

3.1.4 Purity test

3.1.4.1 Protein content

It complies with the determination of protein content (Appendix VI B, method 1).

3.1.4.2 Content of polysaccharide and nucleic acid

The total content of polysaccharide and nucleic acid shall be not more than 0.1 mg/mg of protein.

(1) Polysaccharide content; Dilute the anhydrous glucose standard with physiological saline to make the glucose standard solutions of 0-100 µg/ml. Add 225 ml of sulfuric acid to 75 ml of physiological saline, and dissolve 0.6 g of anthrone in 10 ml of ethanol. Mix above two solutions to obtain the anthrone mixture. Measure accurately 1.0 ml of glucose standard solution at different concentrations and the test sample, respectively, mix each with 4.0 ml of anthrone mixture, and heat in a boiling water bath for 20 minutes. Read the absorbance by spectrophotometry at 620 nm. A regression equation is obtained by plotting the concentration of the glucose standard solution against the corresponding absorbance by minitab or other statistical methods. The polysaccharide content of the test sample is calculated by inserting the absorbance of the test sample into the linear regression equation.

(2) Nucleic acid content; Take 2-3 ml of the test sample and read the absorbance by UV-visible spectrophotometry at 260 nm (Appendix II A). Calculate the content of nucleic acid by using extinction coefficient $E_{1\%}^{1\text{cm}} = 200$.

3.1.5 Potency test

3.1.5.1 Animal test

The TB-PPD standard and the test sample are diluted to three suitable dilutions, respectively. Each of at least four TB sensitized female guinea pigs in white colour weighing 400-600 g, after the hair on the back is removed, shall be injected i. d. with 0.1 ml or 0.2 ml of each dilution of the standard and the test sample on each side of spine in symmetry, respectively. Observe the longitudinal and transverse diameters of induration at the 24th and the 48th hours after injection. Calculate the

sum of diameters (or mean area) of indurations at the 24th and the 48th hours after injection of each dilution of the test sample and the standard and evaluate the ratio, which shall be 0.8-1.2. If the ratio falls short of this value, adjust the dilutions and retest the potency until the ratio is appropriate.

3.1.5.2 Selection of appropriate dilution

The appropriate dilution shall be selected so that the diameters of induration produced 24 hours after injection shall be 8-25 mm. The diameters of indurations caused by sample and TB-PPD standard shall be approximately equal, and the logarithm dose-response curves of three dilutions for both shall be approximately parallel. If the potency of the test sample is different from that of the standard, the test shall be repeated once by the same method. The potency shall be calculated corresponding to the standard and adjustment shall be made. After adjustment, retest the potency by sampling again until the dilution is appropriate.

3.1.6 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.1.7 Test for absence of virulent mycobacteria

3.1.7.1 Test in animals

Each of four TB-PPD (i.d. injection of 0.2 ml containing 10 IU)-negative guinea pigs of the same sex, weighing 300-400 g, shall be injected s. c. with 0.5 ml of the test sample at medial side of thigh. The animals shall be weighed before injection and every 2 weeks after injection. The animals shall not lose weight and shall be autopsied at the end of the 4th week, and no visible pathologic changes of tuberculosis shall be found in any organs. If there is any suspected lesion found in any organs, the smears and the histological sections shall be examined microscopically, and relevant tissues shall be ground, made into a suspension by adding a small quantity of physiological saline and injected into two guinea pigs. The bulk shall be discarded if pathologic changes of tuberculosis are confirmed. If any animal dies of the causes other than tuberculosis within 4 week observation period, it shall be autopsied and examined as above. If more than two animals die, the test shall be repeated.

3.1.7.2 Cultivation of mycobacteria

Each of ten tubes of Lowenstein-Jensen medium is inoculated with 1.0 ml of the test sample and incubated at 37°C for 4 weeks. No growth of mycobacteria shall be found.

3.1.8 Test for sensitizing effect

Divide six guinea pigs that have not been previously used for any tests, each weighing 300-400 g, into test and control groups equally. Each of the three guinea pigs in test group shall be inoculated i. d. with 500 IU of the test sample in a volume of 0.1 ml for 3 times, at intervals of 5 days. Fifteen days after the third injection, the animals in both groups shall be inoculated i. d.

each with 500 IU of the test sample in a volume of 0.1 ml. The animals shall be observed for 3 consecutive days and the reactions of the two groups shall not be significantly different.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3 Control tests on final product

3.3.1 Identity test

Each of at least four TB sensitized guinea pigs shall be injected i. d. with 0.2 ml of the test sample. The mean diameter of induration at the 24th hour after injection of the test sample, shall be not less than 5 mm.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless liquid free of insoluble and foreign matters.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.8-7.4 (Appendix V A).

3.3.3.2 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.3.4 Potency test

Inject i. d. 0.2 ml of the test sample and the TB-PPD standard into each of at least four TB sensitized guinea pigs weighing 400-600 g, respectively. Observe the induration of the injection site at the 24th and the 48th hours after injection, measure the mean diameter (the sum of longitudinal and transverse diameters is divided by 2) of indurations caused by the test sample and the standard at the 48th hour, calculate the accumulative value and evaluate the ratio. The ratio of the test sample to the standard shall be 0.8-1.2.

3.3.5 Sterility test

It complies with the test for sterility (Appendix VIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix VIII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 12 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Purified Protein Derivative of BCG (BCG-PPD)

Purified protein derivative of BCG (BCG-PPD) is a

preparation made by cultivation of *Bacillus* of Calmette and Guérin (BCG), sterilization and filtration, followed by purification. It is used for clinical diagnosis of tuberculosis, the selection of eligibles for BCG vaccination and monitoring of the immune response after BCG vaccination.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

The production area for BCG-PPD shall be separated from the production area for other biologics. All production process of bulk including the inactivation of BCG shall be performed in a completely isolated area. The facilities and apparatus for BCG-PPD production shall be used exclusively. The apparatus made of metal or glass or other materials used directly in production shall be washed and cleaned thoroughly and sterilized. The workers engaged in production of BCG-PPD shall be healthy. They shall be free from tuberculosis and subjected to one or two X-ray examinations of the chest each year. If suspected tuberculosis is revealed in X-ray examination the worker shall be kept away from BCG-PPD production temporarily.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The *Bacillus* of Calmette and Guérin strain D₂PB302 shall be used for the production of BCG-PPD.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lots

The total number of passages for a single production harvest from working seed lot shall not exceed 12.

2.1.4 Control tests on seed lots

2.1.4.1 Microscopic examination of stained smears

The bacteria are in short rod or a slightly curved form with round ends by microscopic examination of stained smears. They shall be acid-fast bacilli.

2.1.4.2 Cultural characteristics

BCG cultivated on Sauton potato medium at 37-39°C shall be crumpled and yellowish colonies and shall be greyish mucoid lawns on ox bile potato medium. The BCG grown in Sauton medium shall form rugous and yellowish pellicles on the surface of the medium.

2.1.4.3 Virulence test

Inject i. p. 1 ml of bacterial suspension (5 mg/ml) into each of four TB-PPD (i. d. injection of 0.2 ml containing 10 IU) -negative guinea pigs of the same sex, weighing 300-400 g. Weigh the animals once a week for 5 weeks, and the body weights of animals shall not decrease. The guinea pigs shall be autopsied 4-5 weeks after injection. Pustules on greater omentum, mesenteric lymph nodes enlargement and splenomegaly may be found. However, no lesions in liver or other organs shall be found visually.

2.1.4.4 Test for the absence of virulent mycobacteria

Inject s. c. 1 ml of bacterial suspension (10 mg/ml) at the medial side of thigh into each of six TB-PPD (i. d. injection of 0.2 ml containing 10 IU)-negative guinea pigs of the same sex, weighing 300-400 g. The animals shall be weighed before injection. The injection site and changes of regional lymph nodes shall be observed once a week following injection. The animals shall be weighed every 2 weeks, and the body weights of animals shall not decrease. Three guinea pigs shall be autopsied at the end of the 6th week, and the other three 3 months after injection for the examination of visceral tuberculosis. No tuberculous lesions shall be found visually. If suspected tuberculous focus is found, stained smears and histological sections shall be examined microscopically. Take samples from some foci, grind and mix well with a quantity of physiological saline. The mixture shall be injected s. c. into two guinea pigs. The BCG strain shall be discarded if the tuberculous lesion is confirmed. Any animal that dies within 3 months shall be subjected to a postmortem examination. If suspected tuberculosis lesion is found, the above mentioned procedures shall be performed. The BCG strain shall be discarded if the tuberculous lesion is confirmed. The test shall be repeated if non-specific death is confirmed and more than one animal die.

2.1.5 Storage of seed lots

The freeze-dried seeds shall be stored at 2-8°C. The liquid seeds shall be stored at or below -70°C.

2.2 Bulk

2.2.1 Working seed for production

The bacterial seed from working seed lot shall be subcultured on Sauton potato medium or modified Sauton synthetic medium. The seed is used for the preparation of bulk.

2.2.2 Production medium

Sauton potato medium, modified Sauton synthetic medium or other approved media shall be used for production.

2.2.3 Inoculation and cultivation

The bacterial seed shall be inoculated onto Sauton potato medium and incubated at 37°C for 2-3 weeks. One additional passage can be done on Sauton potato medium. The well-grown bacterial

pellicles shall be collected from Sauton potato medium and transferred onto the surface of modified Sauton synthetic medium or other suitable media and incubated at 37°C for 1-2 weeks. The well-grown bacterial pellicles shall be selected and transferred onto the surface of modified Sauton synthetic media or other approved media, and incubated at 37°C for 8-10 weeks. The cultures shall be discarded if the submerging, contamination or abnormal growth of pellicle is found during or at the end of incubation.

2.2.4 Sterilization and filtration

At the end of incubation, the cultures shall be sterilized at 121°C for 30 minutes, then filtered to remove the pellicle and bacteria.

2.2.5 Collection and storage

The filtrates shall be collected for purification. If the filtrates need storage, the phenol of 3.0 g/L or other appropriate preservers shall be added. The filtrates shall be stored at 4-8°C, and the storage period shall not exceed 30 days.

2.2.6 Purification

The protein in the filtrate shall be precipitated by trichloroacetic acid and saturated ammonium sulfate. Purify the precipitates by the approved methods and sterilize by filtration to obtain bulk.

2.2.7 Pooling and lyophilization

2.2.7.1 Pooling

The bulk from not more than five runs of purification shall be pooled.

2.2.7.2 Filling and lyophilization

The bulk qualified in control tests shall be diluted to the specified concentration based on the protein content. The bulk is dispensed into each container, and lyophilized immediately after filling.

2.2.8 Control tests on bulk

See Section 3.1.

2.2.9 Storage and storage period

The bulk shall be stored at 2-8 °C. The storage period of the liquid bulk is 60 months starting from the date when the potency test proved qualified. The freeze-dried bulk can be used continually when it is qualified in control tests described in Section 3.1, which shall be carried out every 60 months starting from the date when the potency test proved qualified.

2.3 Final bulk

2.3.1 Formulation

The bulk qualified in control tests shall be diluted to 20 IU/ml or 50 IU/ml with 0.01 mol/L PBS (pH 7.2-7.4 containing 0.0005% polysorbate 80 and 3.0 g/L phenol). The ingredients shall be mixed well during dilution. Samples shall be taken from each bottle for sterility test (Appendix VII A).

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

1 ml or 2 ml per container. Each single human dose is 0.1 ml containing 5 IU of BCG-PPD.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Visual inspection

The freeze-dried bulk looks like a white crisp cake. The reconstituted or liquid bulk shall be a clear, brown liquid, free of insoluble and foreign matters.

3.1.2 Reconstitution time

After adding the water for the injection as stated on the label, the freeze-dried bulk shall be reconstituted completely within 3 minutes.

3.1.3 Moisture content

The moisture content shall be not more than 3.0% (Appendix VII D).

3.1.4 Purity test

3.1.4.1 Protein content

It complies with the determination of protein content (Appendix VI B, method 1).

3.1.4.2 Contents of polysaccharide and nucleic acid

The total content of polysaccharide and nucleic acid shall be not more than 0.1 mg/mg of protein.

(1) Polysaccharide content: Dilute the anhydrous glucose standard with physiological saline to make the glucose standard solutions of 0-100 µg/ml. Add 225 ml of sulfuric acid to 75 ml of physiological saline, and dissolve 0.6 g of anthrone in 10 ml of ethanol. Mix above two solutions to obtain the anthrone mixture. Measure accurately 1.0 ml of glucose standard solution at different concentrations and the test sample, respectively, mix each with 4.0 ml of anthrone mixture, and heat in a boiling water bath for 20 minutes. Read the absorbance by spectrophotometry at 620 nm. A regression equation is obtained by plotting the concentration of the glucose standard solution against the corresponding absorbance by minitab or other statistical methods. The polysaccharide content of the test sample is calculated by inserting the absorbance of the test sample into the linear regression equation.

(2) Nucleic acid content: Take 2-3 ml of the test sample and read the absorbance by UV-visible spectrophotometry at 260 nm (Appendix II A).

Calculate the content of nucleic acid by using extinction coefficient $E_{1\text{cm}}^{1\%} = 200$.

3.1.5 Potency test

3.1.5.1 Animal test

The BCG-PPD (or TB-PPD) standard and the test sample are diluted to three suitable dilutions, respectively. Each of at least four BCG sensitized female guinea pigs in white colour weighing 400-600 g, after the hair on the back is removed, shall be injected i.d. with 0.1 ml or 0.2 ml of each dilution of the standard and the test sample on each side of spine in symmetry, respectively. Observe the longitudinal and transverse diameters of induration at the 24th and the 48th hours after injection. Calculate the sum of diameters (or mean area) of induration at the 24th and the 48th hours after injection of each dilution of the test sample and the standard and evaluate the ratio, which shall be 0.8-1.2. If the ratio falls short of this value, adjust the dilutions and retest the potency until the ratio is appropriate.

3.1.5.2 Selection of appropriate dilution

The appropriate dilution shall be selected so that the diameters of lesions produced 24 hours after injection shall be 8-25 mm. The diameters of induration caused by sample and BCG-PPD (or TB-PPD) standard shall be approximately equal, and the logarithm dose-response curves of three dilutions for both shall be approximately parallel. If the potency of the test sample is different from that of the standard, the test shall be repeated once by the same method. The potency shall be calculated corresponding to the standard and adjustment shall be made. After adjustment, retest the potency by sampling again until the dilution is appropriate.

3.1.6 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.1.7 Test for absence of virulent mycobacteria

3.1.7.1 Test in animals

See Section 2.1.4.4.

3.1.7.2 Cultivation of mycobacteria

Each of ten tubes of Lowenstein-Jensen medium is inoculated with 1.0 ml of the test sample and incubated at 37°C for 4 weeks. No growth of mycobacteria shall be found.

3.1.8 Test for sensitizing effect

Divide six guinea pigs that have not been previously used for any tests, each weighing 300-400 g, into test and control groups equally. Each of the three guinea pigs in test group shall be inoculated i.d. with 500 IU of the test sample in a volume of 0.1 ml for 3 times, at intervals of 5 days. Fifteen days after the third injection, the animals in both groups shall be inoculated i.d. each with 500 IU of the test sample in a volume of 0.1 ml. The animals shall be observed for 3

consecutive days and the reactions of the two groups shall not be significantly different.

3.2 Control test on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product

3.3.1 Identity test

Each of at least four BCG sensitized guinea pigs shall be injected i.d. with 0.2 ml of the test sample. The mean diameter of lesions at the 24th hour after injection of the test sample shall be not less than 5 mm.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless liquid free of any insoluble matters and foreign matters.

3.3.3 Chemical tests

3.3.3.1 pH

The pH shall be 6.8-7.4 (Appendix V A).

3.3.3.2 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.3.4 Potency test

Inject i.d. 0.2 ml of the test sample and the BCG-PPD (or TB-PPD) standard into each of at least four BCG sensitized guinea pigs weighing 400-600 g, respectively. Observe the induration of the injection site at the 24th and the 48th hours after injection, measure the mean diameter (the sum of longitudinal and transverse diameters is divided by 2) of induration caused by the test sample and the standard at the 48th hour, calculate the accumulative value and evaluate the ratio. The ratio of the test sample to the standard shall be 0.8-1.2.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 12 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Purified Protein Derivative of Brucellin (BR-PPD)

Purified protein derivative brucellin (BR-PPD) is a preparation made by cultivation of *Bacillus Brucella*,



sterilization and filtration, followed by purification. It is used for the clinical diagnosis of brucellosis, the selection of eligibles for brucellosis vaccination and monitoring of the immune response after brucellosis vaccination.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus, and animals used for production and control tests shall comply with the requirements set forth in the General Notices.

The production area for BR-PPD shall be separated from the production area for other biologics. All production process of bulk including the inactivation of *Bacillus Brucella*, shall be performed in a completely isolated area. The facilities and apparatus for BR-PPD production shall be used exclusively.

2 Manufacturing

2.1 Bacterial seeds

The bacterial seeds used for production shall comply with the requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name and origin of bacterial strains

The *Brucella suis* type I strain CMCC 55007 (S2) shall be used for production of BR-PPD.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lots

The total number of passages for a single production harvest from working seed lot shall not exceed 12.

2.1.4 Control tests on bacterial seed lots

2.1.4.1 Microscopic examination of stained smears

The bacilli shall be Gram-negative coccobacilli.

2.1.4.2 Biochemical reactions

The bacilli shall be negative in the test for dissociation with 1:1000 trypanflavine, and it shall grow on media containing thionine but not grow on media containing basic fuchsin. The bacilli cultures shall produce a large amount of hydrogen sulfide on liver broth agar slant, and no supplementary carbon dioxide is required for growth.

2.1.4.3 Serological test

The agglutination titer of the bacilli with brucella diagnostic serum shall be more than 1:800 (++++).

2.1.4.4 Phage bacteriolytic test

The cultures shall be lysed by Wb brucella-phage.

2.1.5 Storage of bacterial seed

The freeze-dried seeds shall be stored at 2-8°C. The liquid seeds shall be stored at or below -70°C.

2.2 Bulk

2.2.1 Production medium

The liver infusion agar medium or other approved media shall be used for production.

2.2.2 Inoculum preparation

The bacterial seed shall be inoculated onto liver infusion agar medium and incubated at 37°C for 2-3 days. It can be passaged once more on liver infusion agar medium. The well-grown bacterial pellicles shall be used for inoculation.

2.2.3 Inoculation and cultivation

Inoculate the well-grown bacterial pellicles onto liver infusion agar slant in bottles and incubate at 37°C for 2 days.

2.2.4 Sterilization and centrifugation

At the end of incubation, the cultures shall be sterilized by autoclaving at 121°C for 30 minutes and then centrifuged to remove the bacteria.

2.2.5 Collection and storage

The supernatant shall be collected for purification. If the supernatant need storage, 3.0 g/L phenol or other appropriate preservatives shall be added. The supernatant shall be stored at 4-8°C, the storage period shall not exceed 30 days, and prevented from freezing.

2.2.6 Purification

The protein in the supernatant shall be precipitated by trichloroacetic acid (TCA) and saturated ammonium sulfate. Purify the precipitates by the approved methods and sterilize by filtration to obtain bulk.

2.2.7 Pooling and lyophilization

2.2.7.1 Pooling

The bulk from not more than five runs of purification can be pooled.

2.2.7.2 Filling and lyophilization

The bulk qualified in control tests shall be diluted to a definite concentration based on the protein content. The bulk is dispensed into each container, and lyophilized immediately after filling.

2.2.8 Control tests on bulk

See Section 3.1.

2.2.9 Storage and storage period

The bulk shall be stored at 2-8°C. The storage period of the liquid bulk is 60 months starting from the date when the potency test proved qualified. The freeze-dried bulk can be used continually when it is qualified in control tests described in Section 3.1, which shall be carried out every 60 months starting from the date when the potency test proved qualified.

2.3 Final bulk

2.3.1 Formulation

The bulk qualified in control tests shall be diluted to 20 IU/ml or 50 IU/ml with 0.01 mol/L PBS (pH 7.2-7.4 containing 0.0005% polysorbate 80 and 3.0 g/L phenol). The ingredients shall be mixed well during dilution. Samples shall be taken

from each bottle for sterility test (Appendix XIII A).

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

1 ml or 2 ml per container. Each single human dose is 0.1 ml containing 1 U of BR-PPD.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Visual inspection

The freeze-dried bulk looks like a white crisp cake. The reconstituted or liquid bulk shall be a clear, brown liquid, free of insoluble and foreign matters.

3.1.2 Reconstitution time

After adding the water for the injection as stated on the label, the freeze-dried bulk shall be reconstituted completely within 3 minutes.

3.1.3 Moisture content

The moisture content shall be not more than 3.0% (Appendix VII D).

3.1.4 Purity test

3.1.4.1 Protein content

It complies with the determination of protein content (Appendix VI B, method 1).

3.1.4.2 Contents of polysaccharide and nucleic acid

The total content of polysaccharide and nucleic acid shall be not more than 0.1 mg/mg of protein.

(1) Polysaccharide content: Dilute the anhydrous glucose standard with physiological saline to make the glucose standard solutions of 0-100 $\mu\text{g/ml}$. Add 225 ml of sulfuric acid to 75 ml of physiological saline, and dissolve 0.6 g of anthrone in 10 ml of ethanol, mix above two solutions to obtain the anthrone mixture. Measure accurately 1.0 ml of glucose standard solution at different concentrations and the test sample, respectively, mix each with 4.0 ml of anthrone mixture, and heat in a boiling water bath for 20 minutes. Read the absorbance by spectrophotometry at 620 nm. A regression equation is obtained by plotting the concentration of the glucose standard solution against the corresponding absorbance by minitab or other statistical methods. The polysaccharide content of the test sample is calculated by inserting the absorbance of the test sample into the linear regression equation.

(2) Nucleic acid content: Take 2-3 ml of the test sample and measure the absorbance by UV-visible spectrophotometry at 260 nm (Appendix II A). Calculate the content of nucleic acid by using extinction coefficient $E_{1\%}^{1\text{cm}}=200$.

3.1.5 Potency test

3.1.5.1 Animal test

The BR-PPD standard and the test sample are diluted to three suitable dilutions, respectively. Each of at least four BR sensitized female guinea pigs in white colour weighing 400-600 g, after the hair on the back is removed, shall be injected i. d. with 0.1 ml or 0.2 ml of each dilution of the standard and the test sample on each side of spine in symmetry, respectively. Observe the longitudinal and transverse diameters of induration at the 24th and the 48th hours after injection. Calculate the sum of diameters (or mean area) of induration at the 24th and the 48th hours after injection of each dilution and evaluate the ratio. The ratio of each dilution of the test sample to the standard shall be 0.8-1.2. If the ratio falls short of this value, adjust the dilutions and retest the potency until the ratio is appropriate.

3.1.5.2 Selection of appropriate dilution

The appropriate dilution shall be selected so that the diameters of induration produced 24 hours after injection shall be 10-30 mm. The diameters of induration caused by sample and BR-PPD standard shall be approximately equal, and the logarithm dose-response curves of three dilutions for both shall be approximately parallel. If the potency of the test sample is different from that of the standard, the test shall be repeated once by the same method. The potency shall be calculated corresponding to the standard and adjustment shall be made. After adjustment, retest the potency by sampling again until the dilution is appropriate.

3.1.6 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.1.7 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

3.1.8 Test for sensitizing effect

Divide six guinea pigs, that have not been previously used for any tests, each weighing 300-400 g, into test and control groups equally. Each of the three guinea pigs in test group shall be inoculated i. d. with 20 μg of the test sample in a volume of 0.2 ml for 3 times, at intervals of 5 days. Fifteen days after the third injection, the animals in both groups shall be inoculated i. d. each with 20 μg of the test sample in a volume of 0.2 ml. The animals shall be observed for 3 consecutive days and the local cutaneous reaction of the two groups shall not be significantly different.

3.2 Control tests on final bulk

Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3 Control tests on final product**3.3.1 Identity test**

Each of at least four BR sensitized guinea pigs shall be injected i. d. with 0.2 ml of the test sample. The mean size of induration at the 24th hour after injection of the test sample shall be not less than 5 mm.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless liquid free of any insoluble and foreign matters.

3.3.3 Chemical tests**3.3.3.1 pH**

The pH shall be 6.8-7.4 (Appendix V A).

3.3.3.2 Phenol content

The phenol content shall be not more than 3.0 g/L (Appendix VI M).

3.3.4 Potency test

Inject i. d. 0.2 ml of the test sample and the BR-PPD standard into each of at least four BR sensitized guinea pigs weighing 400-600 g, respectively. Observe the induration of the injection site at the 24th and the 48th hours after injection, calculate the mean diameter (the sum of longitudinal and transverse diameters is divided by 2) of induration caused by the test sample and the standard, and evaluate the ratio of diameters for both. The ratio of the test sample to the standard shall be 0.8-1.2.

3.3.5 Sterility test

It complies with the test for sterility (Appendix XIII A).

3.3.6 Test for abnormal toxicity

It complies with the test for abnormal toxicity (Appendix XIII F).

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 12 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

Schick Test Toxin

Schick test toxin is a preparation made by diluted purified diphtheria toxin. It is used to test the susceptibility of humans to diphtheria.

1 Basic requirements

The facilities, source and subsidiary materials, water, apparatus, and animals used for production

and quality control shall comply with the requirements set forth in the General Notices.

2 Manufacturing**2.1 Bacterial seeds**

The bacterial seeds for production shall comply with the requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.1 Name of bacterial strains

The strain of *Corynebacterium diphtheriae* PW8 or highly toxinogenic and immunogenic strains derived from the PW8 strain shall be used for production.

2.1.2 Establishment of seed lot system

It complies with the Requirements for Bacterial and Viral Strains/Seeds Used for Production and Quality Control of Biologics.

2.1.3 Passage of seed lot

The subculture of the bacterial seed from master seed lot shall not exceed five passages.

2.1.4 Control tests on seed lots**2.1.4.1 Cultural characteristics**

The colonies grown on Loeffler agar medium shall be circular, protuberant and grey in colour with a smooth surface and regular border; those grown on potassium tellurite agar medium shall be shining and grey-black in colour; those grown on blood agar medium shall be opaque, grey in colour, and produce no α -hemolysin.

2.1.4.2 Microscopic examination of stained smears

The bacterium shall be Gram-positive rod with metachromatic granules, and club-shaped swellings at its poles. The bacilli arrange themselves in palisades, X or Y shape.

2.1.4.3 Biochemical reactions

The cultures shall ferment glucose, maltose, galactose and dextrin with production of acid but not gas. They shall not ferment sucrose, mannitol, lactose or soluble starch (Appendix XI V).

2.1.4.4 Specific neutralization test

When the bacterial seed is inoculated onto Elek agar medium, an apparent white precipitation line shall be observed.

2.1.5 Storage of seed lots

The seed lot shall be preserved at 2-8°C.

2.2 Bulk**2.2.1 Working seed lots for production**

The bacterial seed from working seed lot shall be inoculated on blood agar slant and subcultured in toxin producing medium for two to three passages, which shall be then transplanted into seed bottles containing the same medium.

2.2.2 Production medium

Trypsin-digested beef medium or other approved appropriate media shall be used for production.

The media shall not contain any horse meat or other horse tissues.

2.2.3 Crude toxin

The crude toxin is made by the cultivation of the working seed in the toxin-producing medium at appropriate temperature for a period of time, clarified and sterilized by filtration. The potency of crude toxin shall be not less than 150 Lf/ml. Microbial contamination shall be avoided during preparation. If contaminating microorganisms are found by microscopic examination, the cultures shall be discarded.

2.2.4 Toxin purification

2.2.4.1 Ammonium sulfate-active carbon fractionation method or other approved appropriate methods shall be used.

The toxin for purification can be pooled from not more than five batches.

2.2.4.2 Purified toxin shall be sterilized by filtration. Toxins produced with the same seed, same medium, and purified by the same method, which are pooled in one container after sterilization by filtration shall be defined as one batch of bulk. The bulk shall be preserved at 2-8°C for an appropriate time, so as to insure a stable toxicity for preparation of final bulk.

2.2.5 Control tests on bulk

See Section 3.1.

2.3 Final bulk

2.3.1 Formulation

Dilute the bulk to 0.2 MLD/ml with glycerol-gelatin-borate buffer solution or other approved appropriate buffer solutions free from antigenic and allergenic substances.

2.3.2 Control tests on final bulk

See Section 3.2.

2.4 Final product

2.4.1 Defining batches

The Requirements for Defining Batches of Biologics shall apply.

2.4.2 Filling

The Requirements for Filling and Lyophilization of Biologics shall apply.

2.4.3 Specifications

1 ml per container, containing 0.2 MLD of diphtheria toxin.

2.4.4 Packaging

The Requirements for Packaging of Biologics shall apply.

3 Control tests

3.1 Control tests on bulk

3.1.1 Purity test

The purity of bulk shall be not less than 2000 Lf/mg PN (Appendix XI D).

3.1.2 Toxicity test

The toxicity of bulk shall be 25-50 MLD/Lf.

3.2 Control tests on final bulk

3.2.1 Visual inspection

It shall be a clear, colourless or light milky-white liquid free of precipitate and foreign matters.

3.2.2 pH

The pH shall be 7.2-8.2 (Appendix V A).

3.2.3 Potency test

3.2.3.1 Determination of MLD

Inject s.c. 5 ml of the test sample into each of four guinea pigs weighing 240-270 g at abdomen. At least three animals shall die within 72-96 hours after injection and the other one may die earlier or later. This indicates that sample contains about 0.2 MLD/ml of purified diphtheria toxin.

3.2.3.2 Tests for combining power

Dilute the standard of diphtheria antitoxin to 1/75 IU/ml and 1/125 IU/ml respectively to which add an equal volume of the test sample. Incubate at room temperature or 37°C for 30 minutes. Inject i.d. 0.2 ml to each of two rabbits weighing 2-3 kg. Observe the results 72 hours after injection. The injection site of the test sample containing 1/1250 IU of diphtheria antitoxin shall show a redness with a size of 10 mm × 10 mm or larger. No reaction shall occur at the injection site of the test sample containing 1/750 IU of the antitoxin.

3.2.4 Stability test

After exposure to 37°C for 24 hours, the potency of the test sample shall comply with the requirement given in Sections 3.2.3.

3.2.5 Sterility test

It complies with the test for sterility (Appendix VII A).

3.3 Control tests on final product

3.3.1 Identity test

See Sections 3.2.3.2.

3.3.2 Inspection on final containers

The preparation shall be a clear, colourless or light milky-white liquid, free of precipitate and foreign matters.

3.3.3 pH

The pH shall be 7.2-8.2 (Appendix V A).

3.3.4 Potency test

See Section 3.2.3.

3.3.5 Sterility test

It complies with the test for sterility (Appendix VII A)

4 Storage, shipping and validity period

Store and ship at 2-8°C, protected from light. The validity period is 24 months starting from the date of filling of final product.

5 Package inserts

The Requirements for Packaging of Biologics shall apply.

APPENDICES

Contents of Appendices

Appendix I

General Requirements for Preparations	A-6
I A Injections	A-6
I B Suppositories	A-7
I C Eye Preparations	A-8
I D Liquids for External Application	A-9
I E Tablets	A-9
I F Capsules	A-10
I G Ointments, Emulsions	A-10
I H Sprays	A-11
I J Granules	A-12
I K Powders	A-13
I L Nasal Preparations	A-14
I M Gels	A-15

Appendix II

Spectrophotometry	A-16
II A Ultraviolet-visible Spectrophotometry	A-16
II B Atomic Absorption Spectrophotometry	A-17
II C Fluorimetry	A-19
II D Flame Photometry	A-20

Appendix III

Chromatography	A-21
III A Paper Chromatography	A-21
III B High Performance Liquid Chromatography	A-22
III C Gas Chromatography	A-26
III D Size Exclusion Chromatography	A-27

Appendix IV

Electrophoresis	A-30
IV A Cellulose Acetate Film Electrophoresis	A-30
IV B Agarose Electrophoresis	A-30
IV C SDS-Polyacrylamide Gel Electrophoresis	A-31
IV D Isoelectric Focusing Electrophoresis	A-32

Appendix V

V A Determination of pH Value	A-35
V B Test for Visible Particles	A-36
V C Determination of Disintegration	A-37
V D Disintegration Test for Suppositories and Vaginal Tablets	A-39
V E Test for Tablet Friability	A-40
V F Test for Minimum Fill	A-41
V G Determination of Particle Size	A-41
V H Determination of Osmolality	A-42

Appendix VI

VI A Determination of Nitrogen	A-44
VI B Determination of Protein Content	A-45
VI C Determination of Sialic Acid Content	A-46
VI D Determination of Residual Ethanol Content	A-47
VI E Determination of Free Histamine Phosphate in Human Histamine Immunoglobulin	A-48
VI F Determination of O-Acetyl Content	A-48
VI G Determination of Residual Polyethylene Glycol Content	A-49
VI H Determination of Residual Polysorbate 80 Content	A-50
VI I Determination of Residual Glutaraldehyde Content	A-50
VI J Determination of Tributylphosphate Content	A-51
VI K Determination of Sodium Caprylate Content	A-51
VI L Determination of Free Formaldehyde Content	A-52
VI M Determination of Phenol Content	A-53
VI N Determination of Metacresol Content	A-53
VI O Determination of Trichloromethane Content	A-54
VI P Determination of Saccharides and Sugar Alcohol Content in Human Blood Products	A-54
VI Q Determination of Polymer Content in Human Albumin	A-55
VI R Determination of IgG Monomer and Dimer in Human Immunoglobulins	A-55

Appendix VII

VII A Determination of Phosphorus Content	A-57
VII B Determination of Thimerosal Content	A-57
VII C Determination of Ammonium Sulfate Content	A-58
VII D Determination of Moisture Content	A-58
VII E Determination of Sodium Bisulfite Content	A-59
VII F Determination of Aluminium Hydroxide (or Aluminium Phosphate) Content	A-60
VII G Determination of Sodium Chloride Content	A-61
VII H Determination of Citrate Content	

.....	A-61
VII I Determination of Potassium Content A-62
VII J Determination of Sodium Content A-62
VII K Determination of Residual Aluminium Content in Human Albumin A-63
VII L Determination of Loss on Drying A-63
VII M Determination of Total Solid A-64

Appendix VII

VII A Immunoblot A-65
VII B Immunodot A-65
VII C Double Immunodiffusion A-66
VII D Immuno-electrophoresis A-67
VII E Peptide Mapping A-67
VII F Determination of F(ab) ₂ Content in Antitoxin A-68
VII G Determination of Molecular Size for Group A Meningococcal Polysaccharide A-68
VII H Determination of Molecular Size for Typhoid Vi Polysaccharide A-69

Appendix IX

IX A Determination of Residual Antibiotics A-71
IX B Determination of Residual Extraneous DNA A-71
IX C Determination of Residual Host Bacterial Protein (<i>E. coli</i>) A-73
IX D Determination of Residual Host Bacterial Protein (<i>Pseudomonas</i>) A-74
IX E Determination of Residual Host Yeast Protein A-75
IX F Determination of Prekallikrein Activator Content A-76
IX G Test for Losing Rate of Plasmid A-77
IX H Test for Nucleotide Sequence of SV40 A-77
IX I Test for Blood Group A-like Substance A-78
IX J Test for Anti-A and Anti-B Hemagglutinins A-78
IX K Test for Anticomplement Activity A-79
IX L Determination of Residual Murine IgG A-81
IX M Test for Reverse Transcriptase Activity A-82
IX N Test for Human Thrombin Activity A-83
IX O Test for Activated Coagulation Factor Activity A-83
IX P Determination of Heparin Content A-84
IX Q Determination of Human Erythrocyte Antibody A-84
IX R Determination of Human Platelet Antibody A-85

Appendix X

X A <i>In vitro</i> Test for Relative Potency of Recombinant Hepatitis B Vaccine (Yeast) A-87
X B <i>In vivo</i> Test for Biological Activity of Recombinant Human Erythropoietin A-87
X C Biological Activity Test for Interferon A-88
X D Biological Activity Test for Recombinant Human Interleukin-2 A-89
X E Biological Activity Test for Recombinant Human Granulocyte Colony-stimulating Factor A-89
X F Biological Activity Test for Recombinant Human Granulocyte/Macrophage Colony-stimulating Factor A-90
X G Biological Activity Test for Recombinant Bovine Basic Fibroblast Growth Factor A-91
X H Biological Activity Test for Recombinant Epidermal Growth Factor A-92
X I Biological Activity Test for Recombinant Streptokinase A-93
X J Potency Test for Human Coagulation Factor II A-93
X K Potency Test for Human Coagulation Factor VII A-94
X L Potency Test for Human Coagulation Factor IX A-94
X M Potency Test for Human Coagulation Factor X A-95
X N Potency Test for Human Coagulation Factor VIII A-96
X O Potency Test for Diphtheria Antibody in Human Immunoglobulin A-96
X P Test for Fc Function in Human Immunoglobulin A-97
X Q Potency Test for Anti-human T Lymphocyte Immunoglobulin (E-rosette Formation-inhibition Test) A-98
X R Potency Test for Anti-human T Lymphocyte Immunoglobulin (Lymphocytotoxicity Test) A-99

Appendix XI

XI A Potency Test for Rabies Vaccine for Human Use A-101
XI B Potency Test for Adsorbed Tetanus Vaccine A-101
XI C Potency Test for Adsorbed Diphtheria Vaccine A-102
XI D Determination of Flocculation Unit of Toxoid A-103
XI E Potency Test for Diphtheria Antitoxin A-104
XI F Potency Test for Tetanus Antitoxin A-105
XI G Potency Test for Gas-gangrene Antitoxins A-105
XI H Potency Test for Botulinum

	Antitoxin	A-106
XI I	Potency Test for Snake Antivenins	A-107
XI J	Potency Test for Rabies Antiserum	A-108
XI K	Determination of IgG Content	A-110
XI L	Test for Neurovirulence in Monkeys	A-110

Appendix XII

XII A	Sterility Test	A-113
XII B	Test for Mycoplasma	A-116
XII C	Test for Adventitious Viruses	A-118
XII D	Pyrogen Test	A-119
XII E	Test for Bacterial Endotoxin	A-120
XII F	Test for Abnormal Toxicity	A-124
XII G	Microbial Limit Tests	A-125
XII H	Test for Murine Virus	A-134

Appendix XIII

XIII A	Test Requirements for SPF Chicken Embryos	A-137
XIII B	Test Requirements of Microbes for Laboratory Animals	A-137
XIII C	Test Requirements of Parasites for Laboratory Animals	A-139
XIII D	Test Requirements for Calf Serum	A-140

Appendix XIV

	Culture Media for Biochemical Reactions of Bacteria and Test Method	A-141
--	---	-------

Appendix XV

	Sterilization	A-146
--	---------------------	-------

Appendix XVI

	Names, Symbols and Atomic Weights of Elements	A-150
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Appendix I General Requirements for Preparations

The General Requirements are applicable to the biologics for therapeutic use, including blood products, immune sera, cytokines, monoclonal antibodies, immunoregulators, probiotics, etc. The biologics for prophylactic use shall comply with the requirements in relevant monographs.

I A Injections

Injections Injections are sterile preparation made from drug substances and appropriate stabilizers or other subsidiary materials, intended for parenteral administration into the body, including solutions, emulsions, suspensions as well as sterile freeze-dried products which shall be reconstituted with sterile solvents to make solutions or suspensions before use.

The injections are in forms of liquid and sterile powder.

Solutions for injection Solutions for injection are sterile, aqueous solutions, emulsions or suspensions intended for parenteral administration into the body. They may be used for subcutaneous, intradermal, intramuscular and intravenous injections, as well as intravenous drip. The solutions in large volumes (not less than 50 ml in general, unless otherwise specified) for intravenous drip are also defined as intravenous infusion.

Sterile powders for injection (freeze-dried products are considered as powders for injection) Sterile powders for injection refer to the sterile powder or mass which is reconstituted with suitable sterile solvents to make clear solutions or homogeneous suspensions before use. After reconstitution they can be used for injection. They can also be used for intravenous drip after addition of intravenous infusion.

The production and storage of injections shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.
2. The solvents used must be safe and innocuous and shall not affect the therapeutic efficacy and quality of the injections. They are classified as aqueous and nonaqueous solvents in general.
 - (1) Water for injection is the most commonly used aqueous solvent, 0.9% sodium chloride solution

or other aqueous solutions may also be used.

(2) The sterile solvents for freeze-dried preparation are also defined as diluent for injection use. The most commonly used diluents are sterile water for injection and sodium chloride injection, which shall comply with the relevant requirements in the Pharmacopeia (Volume II). Other diluents for injection use, unless otherwise specified, shall be tested for sterility, pyrogens or bacterial endotoxins and abnormal toxicity, and shall comply with the requirements.

3. Suitable additives may be added according to the nature of the preparations during formulation of injections. The commonly used additives include pH adjusting solutions, stabilizers, solubilizers, antioxidants, preservatives etc. The additives used shall not affect the therapeutic efficacy of injections or interfere the results of tests.

The concentrations of preservatives used for injections shall be controlled. The concentration of phenol used as a preservative shall be not more than 0.5%, and that of thimerosal used shall be not more than 0.01%. Unless otherwise specified, the injections for intravenous use shall be free from any preservatives, and the solubilizers shall be used with caution. The antioxidants, such as vitamin C, vitamin E and sodium thiosulfate, may enhance the stability of biologically active substances after lyophilization during storage. The excipients, such as sucrose, sorbitol and lactose, may make the biologically active substances to develop definite forms during and after lyophilization and protect the substances from loss due to spread.

4. The containers commonly used for injections include glass ampoules, glass bottles, plastic bottles, prefilled syringes etc. All the containers, as well as their stoppers, shall comply with the relevant national standards.

5. The actual filling quantity of injections shall comply with the Requirements for Filling and Lyophilization of Biologics.

A multidose container, unless otherwise specified, shall contain not more than 10 injecting doses.

6. The container after filling shall be immediately sealed by fusion or sealed tightly with stopper. If the containers are required to be sealed under vacuum or after filling with nitrogen, the air in containers shall be expelled at first. For the temperature-sensitive preparations, the temperature during filling and sealing shall be controlled, and

the containers after sealing shall be stored at the specified temperature immediately.

7. The freeze-dried preparations for injection after filling shall be lyophilized timely under suitable conditions according to the Requirements for Filling and Lyophilization of Biologics. The residual moisture content in preparations after lyophilization shall comply with the requirements in relevant monographs.

Unless otherwise specified, after being sealed by fusion or sealed tightly with stopper, the containers of injections shall be subject to an inspection of leakage by reduced pressure or by other suitable methods.

8. Unless otherwise specified, the injections shall be stored and shipped at 2-8°C, protected from light.

Filling quantity Unless otherwise specified, the filling quantity of injection shall comply with the following requirements.

Take 5 containers of test sample if the labelled quantity is 2 ml or less; 3 containers if the labelled quantity is more than 2 ml but not more than 50 ml. Open the containers with caution to avoid any loss of the contents. Take up individually the contents of each container into a dry syringe, then transfer the contents in the syringe into a calibrated cylinder, and measure the volume at room temperature. The cylinder shall be selected to make the volume to be measured account for at least 40% of its specified volume. For injections of suspension, shake the containers thoroughly before removing the contents, then determine as mentioned above. The filling quantity in each container shall be not less than the labelled quantity of the injection.

The injections with labelled quantities of more than 50 ml shall comply with the test for minimum fill (Appendix V F).

Weight variation Unless otherwise specified, the weight variation of freeze-dried preparations for injection shall comply with the following requirements.

Procedure Take 5 containers of test sample, remove their label and aluminium cover, wash the outside wall with ethanol and dry thoroughly. Open the containers with caution to avoid foreign matter such as glass bits falling into container and weigh accurately the individual container immediately. Remove the content, wash the container with water or ethanol, dry under a suitable condition and weigh accurately again. Calculate the weight of each container and the average weight of 5 containers. The weight variation of each container shall not deviate from the average weight by a percentage greater than that shown in the following table. If the weight variation of contents of one container does not comply with the above requirements, repeat the test with another 10 containers of test sample. All of them shall comply with the requirements listed in the following table.

Average weight	Weight variation limit
0.05 g or less	±15%
More than 0.05 g to 0.15 g	±10%
More than 0.15 g to 0.50 g	±7%
More than 0.50 g	±5%

Visible particles Comply with the test for visible particles (Appendix V B), unless otherwise specified.

Sterility Comply with the test for sterility (Appendix III A).

I B Suppositories

Suppositories are solid preparations made of drug substances, in liquid or dry powder form, and suitable bases, intended for rectal, vaginal and urethral administration.

The suppositories for rectal use are made in shapes of torpedo, cylinder or circular cone etc; the suppositories for vaginal use are in shapes of duck mouth, ball or egg; the suppositories for urethral use are in shapes of club in general.

The production and storage of suppositories shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.

2. Semisynthetic fatty acid glycerides, cocoa butter, polyoxyethylene stearate, fatty acid ester of polyoxyethylene sorbitan, hydrogenated vegetable oil, glycerinated gelatin, polyethylene glycols and other suitable materials are usually used as the bases of suppository.

Oily materials such as cocoa butter shall not be used as the bases of suppository for vaginal administration since they can not be absorbed in vagina and may form residues. The suppositories for vaginal administration are usually prepared with water-soluble bases or those that can be mixed well with water.

3. Solid drug substances used for preparation of suppositories, unless otherwise specified, shall be prepared into fine powder by suitable methods before use. Suppositories may be made in different shapes according to their usage.

4. Surfactants, diluents, absorbents, lubricants, preservatives etc. may be added if necessary.

5. The drug substances and bases used for suppositories shall be mixed well. The suppositories shall be smooth in appearance and uniform in texture. Suppositories shall be non-irritating and molten, soften or dissolved when inserted into the canals and shall be miscible with secretion, then gradually release the medicaments to exert local or systemic effects. Suppositories shall be of suitable hardness to prevent from deformation during

packaging or storage.

6. Packing materials or containers used for suppositories shall be non-toxic and do not interact physically or chemically with the medicaments or bases.

7. Unless otherwise specified, suppositories shall be stored and shipped at 2-8°C, protected from light, heat and damp to avoid deformation, mould contamination or deterioration.

The suppositories shall be subject to the following tests.

Weight variation Weight variation of suppositories shall comply with the following requirements.

Average weight	Weight variation limit
1.0 g or less than 1.0 g	±10%
More than 1.0 to 3.0 g	±7.5%
More than 3.0 g	±5.0%

Procedure Weigh together ten suppositories accurately and calculate the average weight, then weigh accurately each of the ten suppositories. Not more than one of the individual weight shall deviate from the average weight by more than the weight variation limit shown in the table, and none shall deviate by more than twice of that percentage.

Disintegration Comply with the disintegration test for suppositories (Appendix V D), unless otherwise specified.

Microbial limit Comply with the microbial limit tests (Appendix III G), unless otherwise specified.

I C Eye Preparations

Eye preparations are liquid or semisolid eye preparations intended for administration upon the eyeball for therapeutic use. Liquid preparations include eye drops, eye washings and intra-ocular injections; semisolid preparations include eye ointments, eye emulsions and eye gels.

The production and storage of eye preparations shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.

2. Unless otherwise specified, eye drops shall be isotonic with tears and shall be tested for osmolality.

3. Unless otherwise specified, the filling quantity of eye drops in each container shall be not more than 10 ml.

4. The bases of eye ointments shall be filtrated and sterilized. Eye ointments shall be homogeneous, fine, non-irritating and easy to be spread to eyes

for dispersion and absorption of medicaments.

5. The containers shall not be broken easily. They shall be cleaned and sterilized, and their transparency shall not interfere the test for visible particles.

6. Unless otherwise specified, eye preparations shall be stored and shipped at 2-8°C in tightly closed containers, protected from light.

7. Multidose eye preparations shall be used within 4 weeks after opening the container.

The eye preparations shall be subject to the following tests.

Visible particles Unless otherwise specified, eye drops shall comply with the test for visible particles (Appendix V B).

Metal particles Unless otherwise specified, semi-solid eye preparations shall comply with the test for metal particles.

Procedure Take 10 containers of test sample, transfer the content of each container as completely as possible into separate culture dishes which are 6 cm in diameters with flat bottoms and to make the test sample free from gas bubbles or visible foreign particles. Cover the dishes, heat at 85°C for 2 hours and make the test sample distributing uniformly, cool at room temperature until it is congealed. Invert each dish on the stage of a suitable microscope. Each dish is illuminated from above direction by a spotlight placed at an angle of 45° to the bottom of the plate. Examine at a 30 magnification and count the lustrous metal particles of 50 µm or greater in dimension. Not more than one out of 10 containers shall be found to contain more than 8 metal particles, and not more than 50 particles shall be found in total in the 10 containers. If the preparation fails to pass the test, repeat the test with another 20 containers and the results from both tests shall be calculated together. The preparation passes the test if not more than 3 containers are found each containing more than 8 metal particles, and not more than 150 particles are found in total in the 30 containers.

Variation of weight or filling quantity Unless otherwise specified, the semisolid eye preparations supplied in singledose containers shall comply with the following requirements.

Procedure Weigh the content of each of 20 containers and calculate the average weight. It shall be not more than 2 containers with the excess of content by ±10% of average weight, and none with the excess of content by ±20% of average weight.

Filling quantity Unless otherwise specified, the filling quantity of single-dose liquid eye preparations shall comply with the following requirements. Take the test sample from each of 10 containers and measure the volume of individual content. The filling quantity in each container shall be not less

than the labelled quantity.

The multidose eye preparations shall comply with the test for minimum fill (Appendix V F).

Microbial limit Comply with the microbial limit test (Appendix XII G), unless otherwise specified. Where the sterility tests on eye preparations are required, the microbial limit test may not be carried out.

I D Liquids for External Application

Liquids for external application are sterile clear solutions prepared with drug substances and suitable stabilizers or other subsidiary materials, intended for application to the surface of wound.

The production and storage of liquids for external application shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.
2. The medicaments shall be absorbed by the surface of wound. Apply the solutions onto wound parts slightly with cotton sticks or other soft materials, which shall be clean, sterilized and free from contamination of microorganisms.
3. The liquids for external application shall be free from abnormalities such as rancidity, abnormal odor and discolouration. Suitable preservatives or antioxidants may be added to solutions if necessary.
4. Unless otherwise specified, liquids for external application shall be stored and shipped at 2-8°C, protected from light.

Filling quantity Comply with the test for filling quantity (Appendix I A), unless otherwise specified.

Sterility Comply with the test for sterility (Appendix XIII A).

I E Tablets

Tablets are solid preparations, in round or other shapes, obtained by compressing the uniform mixture of drug substances of biologics, in a form of dry powder, and suitable subsidiary materials. The drug substances may contain one or more active ingredients.

They are mainly conventional oral tablets, or effervescent tablets, enteric coated tablets etc.

Conventional oral tablets Conventional oral tablet are prepared by compressing uniform mixture of drug substances and suitable subsidiary materials.

Most of uncoated tablets are conventional oral tablets. The weight of each tablet is 0.1 - 0.5 g in general.

Effervescence tablets Effervescence tablets refer to the tablets containing sodium bicarbonate and organic acids, which release carbon dioxide in effervescent appearance when dissolved in water.

The ingredients in effervescent tablets shall be freely soluble. They shall be dissolved after water is added and bubbles are produced. The organic acids used are citric acid, tartaric acid and fumaric acid in general.

Enteric coated tablets Enteric coated tablets are the tablets coated with enteric coating materials. Tablets may be coated with enteric coating materials to prevent the active ingredients from decomposition and losing their effectiveness, irritation to stomach or to control the active ingredient target releasing in the intestinal fluid. Colon specific tablets are film-coated tablets intended for treatment of colon diseases.

The production and storage of tablets shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.
 2. The drug substances shall be mixed well with the subsidiary materials, and the contents of medicaments shall be accurate. Tablets containing drug substances in small amount shall be dispersed uniformly by an appropriate method.
 3. Tablets containing volatile, thermolabile or photosensitive substances shall be protected from light and heat during processing to avoid the loss of ingredient or effectiveness.
 4. The moisture content in final bulk before compressing tablets shall be controlled to meet the requirements in relevant monographs. Correctives, aromatics and colouring agents may be added according to the actual need.
 5. Tablets may be coated with a film in order to enhance the stability, mask unpleasant tastes and improve the appearance.
 6. Tablets shall have a clean, smooth and uniformly coloured surface. They shall be of suitable hardness and wearing-resistance. Unless otherwise specified, the uncoated tablets shall comply with the test for friability to resist wearing or cracking during packaging, storage and shipping.
 7. Unless otherwise specified, the tablets shall be stored and shipped in tightly closed containers at 2-8°C or in a suitable humidity. The tablets shall be subject to the following tests.
- Weight variation** Weight variation of tablets shall comply with the following requirements.

Average or labelled weight	Weight variation limit
Less than 0.30 g	$\pm 7.5\%$
0.30 g or more	$\pm 5.0\%$

Procedure Weigh accurately 20 tablets and calculate the average weight; then weigh individually each of the 20 tablets (compare the weight of each tablet with the labelled weight for the tablets without content determination). Not more than 2 of the individual weights shall deviate from the average weight by more than the weight variation limit shown in the table, and none shall deviate by more than twice the limit.

The film coated tablets shall comply with the test for weight variation after being coated.

Disintegration Unless otherwise specified, comply with the test for disintegration (Appendix V C).

Where dissolution test, drug release test or disintegration test for suppositories is required, disintegration test may not be carried out.

Microbial limit Comply with the microbial limit test (Appendix XIII G), unless otherwise specified. Where the test for contaminating microorganisms on tablets is required, the microbial limit test may not be carried out.

I F Capsules

Capsules are solid preparations consisting of dry powder of drug substances and suitable subsidiary materials, enclosed in hard or soft shells. They usually contain one or more active ingredients. Capsules include hard capsules (generally called as capsules) and enteric capsules etc.

Hard capsules Hard capsules are prepared by enclosing in a capsule shell the uniform powders, granules, small tablets or small pills made of drug substances and suitable subsidiary materials.

Enteric capsules Enteric capsules are prepared by treating hard capsules with medicinal high molecular materials or by other suitable methods. They may be prepared with suitable enteric materials or by enclosing in a capsule shell the granules or small pills coated with enteric materials. The capsules shall be insoluble in gastric fluid, but can be disintegrated in intestinal fluid to release active ingredients.

The production and storage of capsules shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.
2. The active ingredients or subsidiary materials enclosed in capsules shall not cause the deterioration of shells.

3. Capsules shall have a clean and smooth surface and be well shaped without adhesion, deformation, leakage or breakage. Capsules shall not have abnormal odor.

4. Unless otherwise specified, capsules shall be stored and shipped at 2-8°C in tightly closed containers.

The capsules shall be subject to the following tests.

Weight variation Weight variation of capsules shall comply with the following requirements.

Average weight	Weight variation limit
Less than 0.30 g	$\pm 10\%$
0.30 g or more	$\pm 7.5\%$

Procedure Weigh accurately 20 capsules, unless otherwise specified. Open each capsule and remove the content (without loss of capsule shell) as completely as possible (for hard capsules, clean the shell with a small brush). Weigh accurately the shell of each capsule and calculate the weight of content of each capsule and the average weight of the capsules. Not more than 2 capsules of the individual weight shall deviate from the average weight by more than the weight variation limit shown in the table, and none shall deviate by more than twice of that percentage.

Disintegration Comply with the test for disintegration (Appendix V C), unless otherwise specified.

Microbial limit Comply with the microbial limit test (Appendix XIII G), unless otherwise specified. Where the test for contaminating microorganisms on capsules is required, the microbial limit test may not be carried out.

I G Ointments, Emulsions

Ointments are semisolid preparations made of drug substances, in liquid or dry powder form, and suitable bases which are mainly lipophilic or water-soluble, and intended for external use. Ointments may be divided into solution and suspension ointments according to the phases of medicaments dispersed in bases. Solution ointments are the ointments in which the medicaments dissolve or melt into the simple or compound bases; suspension ointments are the ointments in which the fine powders of the medicaments are dispersed into the bases evenly.

Emulsions are homogeneous semisolid preparations made of drug substances, in liquid or dry powder form, dispersed or dissolved in emulsion bases, and intended for external use. They may be divided into oil-in-water and water-in-oil emulsions based on the

different bases used.

The production and storage of ointments and emulsions shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.

2. The bases of ointments and emulsions shall be selected according to the characteristics of dose forms, properties, therapeutic effects and stabilities of preparations. The different kind of bases may be mixed for use.

The bases of ointments include lipophilic and water-soluble ones. Lipophilic bases are mainly Vaseline, paraffin, liquid paraffin, silicone oil, beeswax, stearic acid and wool fat etc. Water-soluble bases are mainly polyethylene glycols.

The bases of emulsions include oil-in-water and water-in-oil emulsifying agents. The oil-in-water emulsifying agents are mainly sodium soap, triethanolamine soap, aliphatic ethanol sodium sulfate (sodium dodecyl sulfate) and polysorbate. The water-in-oil emulsifying agents are mainly wool fat, monoglyceride, fatty alcohol etc.

3. The bases of ointments shall be homogeneous, fine and smooth and nonirritating to skin or mucous membranes. Insoluble solid medicaments in suspension ointments shall be powdered finely by suitable method in advance to ensure the particle size in compliance with the relevant requirements.

4. If necessary, moisturizers, preservatives, thickeners, antioxidants and transdermal accelerants may be added to the ointments or emulsions.

5. When ointments or emulsions are to be used for extensive burns and severely injured skin, both bases and preparations shall be sterilized before use.

6. Ointments and emulsions shall be of proper viscosity. They shall be applied easily to the skin or mucous membrane but shall not be melted. The viscosity shall only change a little with different seasons.

7. Ointments and emulsions shall show no evidence of deterioration such as rancidity, abnormal odor, discolouration, hardening or separation of oil and water.

8. The immediate packaging materials for ointments and emulsions shall not interact physically or chemically with the medicaments or with the bases. The immediate packaging materials for sterile preparations shall be sterile.

9. Unless otherwise specified, ointments and emulsions shall be stored and shipped at 2-8°C in tightly closed containers, protected from light. Ointments and emulsions shall be subject to the following tests.

Particle size Unless otherwise specified, spread a quantity of the suspension ointments onto three microscope slides separately with a thin layer whose area is equivalent to the covered slide. Carry out the test for particle size (Appendix V G, method 1), and no particle of greater than 180 μm in dimension shall be observed.

Filling quantity Comply with the test for minimum fill (Appendix V F).

Microbial limit Comply with the microbial limit test (Appendix XIII G), unless otherwise specified. Where the tests for sterility on ointments and emulsions are required, the microbial limit test may not be carried out.

Sterility Unless otherwise specified, ointments and emulsions used for treatment of extensive burn and severely injured skin shall comply with the test for sterility (Appendix XIII A).

I H Sprays

Sprays are liquid preparations made from drug substances with suitable stabilizers or subsidiary materials, which are filled into special devices. The preparations are released in the form of aerosol with the aid of atomizing devices, or by means of high pressure gas, supersonic vibration or other suitable methods. They are also called spray for external use and classified to quantitative and non-quantitative preparations based on quantitative administration of drug or not. The production and storage of liquids for nebulization shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.

2. The temperature during production shall be controlled, and the produced liquids for nebulization shall be stored at the specified temperature immediately to minimize the adverse effect on the active ingredients of drug.

3. During processing of liquids for nebulization, solvents, solubilizers, antioxidants, surfactants or other auxiliary substances may be added if necessary. The added substances shall be non-irritating to skin.

4. The solution sprays shall be clear. Emulsion sprays shall be dispersed uniformly in the vehicle. Suspension sprays shall be a stable suspension prepared by mixing drug substances and additives thoroughly.

5. All the component units of the atomizing device shall be nontoxic, nonirritating, stable, and compatible with the contents. Unless otherwise specified, the sprays shall be

stored and shipped at 2-8°C, protected from light. The sprays shall be subject to the following tests.

Total number of deliveries per container For multidose sprays, the total number of deliveries per container shall comply with the requirements of the following test.

Procedure Take 4 containers of test sample and remove their covers. Weigh each container accurately (W_1), shake thoroughly, and discharge 10 doses to a container containing a quantity of absorbent solution in a fume hood following the instructions. Wash the mouthpieces with a suitable solvent, dry and weigh each container accurately (W_2). Shake, and continuously discharge 10 doses to the above container. Wash the mouthpieces with a suitable solvent, dry sufficiently, and weigh each container accurately (W_3). Open the storage container, remove the drug liquid, wash each container with a suitable solvent, dry and weigh each container accurately (W_4). Calculate the total number of delivery per container by the following formula. The total number of delivery per container shall be not less than the labelled number of delivery.

$$\text{Total number of delivery per container} = 10 \times (W_1 - W_4) / (W_2 - W_3)$$

Delivery amount in a dose Unless otherwise specified, quantitative sprays shall comply with the requirements of the following test.

Procedure Take 4 containers of test sample and remove their covers, discharge several doses following the instructions, blot the containers and weigh accurately. Continuously discharge 3 doses, blot the containers each time after discharging and weigh each container accurately, then calculate the delivery amount in a dose based on the 3 doses. Continuously discharge 10 doses, blot the containers and weigh each container accurately. Repeat the procedure and calculate the delivery amount in a dose based on the other 3 doses. Continuously discharge 10 doses, blot the containers and weigh each container accurately. Repeat the procedure and calculate the delivery amount in a dose based on another 4 doses. Calculate the average delivery amount per spray based on the 10 deliveries. Unless otherwise specified, the result shall be not less than 80% and not more than 120% of the labelled amount in one delivery.

Where the test for content of active ingredient per spray is required, the test for delivery amount in a dose may not be carried out.

Content of active ingredient in a unit spray Unless otherwise specified, quantitative sprays shall comply with the requirements of the following test.

Procedure Take one container of test sample, discharge 5 deliveries following the instructions, wash the mouthpiece with a suitable solvent and dry sufficiently. Discharge 10 or 20 deliveries (at

the interval of 5 seconds and with slow shaking) and collect the content into a quantity of absorbent solution. Transfer the combined solution to a suitable volumetric flask, dilute with the solvent to volume and shake up. Determine the content and divide the result by 10 or 20 to calculate the content of active ingredient in a unit spray. The result shall be not less than 80% and not more than 120% of the labelled amount of ingredient in a unit spray.

Weight variation Unless otherwise specified, the weight variation for single-dose sprays shall comply with the following requirements.

Average weight	Weight variation limit
Less than 0.30 g	±10%
0.30 g or more	±7.5%

Procedure Unless otherwise specified, take 20 containers of test sample and calculate the weight of content of each container and the average weight by the methods given in relevant monographs. Not more than 2 of the individual weights shall deviate from the average weight by more than the weight variation limit shown in the table, and none shall deviate by more than twice of that percentage.

Filling quantity Multidose preparations shall comply with the test for minimum fill (Appendix V F).

Microbial limit Comply with the microbial limit test (Appendix XII G), unless otherwise specified. Where the sterility test on sprays is required, the microbial limit test may not be carried out.

Sterility The sprays intended to be applied to burns, serious wounds or ulcer shall comply with test for sterility (Appendix XII A).

I J Granules

Granules are dry granular preparations with an appropriate particle size, which are made of dry powders of drug substances and suitable subsidiary materials. The drug substances may contain one or more active ingredients. Granules are intended for oral administration, which may be classified into soluble granules (generally called as granules), suspension granules, enteric granules etc.

Suspension granules Suspension granules are dry granular preparations with an appropriate particle size, which are made of insoluble drug substances and suitable subsidiary materials. They are intended for oral administration, which are dispersed in water or other suitable solvents on shaking to form a suspension.

Unless otherwise specified, suspension granules shall comply with the dissolution test.

Enteric granules Enteric granules are the preparations made of granules coated with enteric materials or made by other suitable methods.

Enteric granules are gastro-resistant and modified-release granules that are intended to resist the gastric fluid and to release active ingredients in intestinal fluid to avoid the decomposition of medicaments in stomach and the irritation to stomach and control the release.

Unless otherwise specified, enteric granules shall be subject to the drug release test.

The production and storage of granules shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.
2. The drug substances and subsidiary materials shall be mixed well. Temperature shall be controlled during the manufacture of volatile or heat-labile drug substances. The drug substances unstable to light shall be protected from light.
3. Granules shall be dry, uniform in particle size and colour, and show no evidence of moisture absorbing, softening, agglomeration or deliquescence.
4. If necessary, some suitable auxiliary substances such as flavoring agents, aromatics, colouring agents, dispersing agents and preservatives etc. may be added to granules during the manufacture.
5. Unless otherwise specified, granules shall be preserved in well-closed containers and stored and shipped at 2-8°C in a dry place, and protected from light.

The granules shall be subject to the following tests.

Size of granules Comply with the test for particle size (Appendix V G), unless otherwise specified. The total weight of granules including those can not pass through sieve No. 1 (2000 μm) and those can pass through sieve No. 5 (180 μm), shall be not more than 15% of the quantity of test sample.

Loss on drying Comply with the test for loss on drying (Appendix VII L), unless otherwise specified. The test sample shall be dried to constant weight at 105°C. Granules containing sugar shall be dried at 80°C under a reduced pressure and shall lose not more than 2.0% of its weight.

Dispersion Unless otherwise specified, soluble granules shall comply with the requirements of the following test.

To 10 g of granules, add 200 ml of hot water and stir for 10 minutes. Soluble granules shall be dissolved completely or show slight turbidity without foreign matter.

Where the tests for dissolution or drug release on

suspension granules are required, the test for dispersion may not be carried out.

Weight variation The weight variation limit of single-dose package of granules shall comply with the following requirements.

Average or labelled weight	Weight variation limit
1.0 g or less than 1.0 g	$\pm 10\%$
More than 1.0 g to 1.5 g	$\pm 8\%$
More than 1.5 g to 6.0 g	$\pm 7\%$
More than 6.0 g	$\pm 5\%$

Procedure Take 10 packs (bottles) of test sample, remove the wrapper, and weigh accurately the content of each separately. Calculate the weight of each pack (bottle) and the average weight. Compare the weight of each pack (bottle) with the average weight (for granules without content determination, compare the weight of each pack (bottle) with the labelled weight). Not more than 2 individual values shall exceed the limit, and none shall exceed by doubling the limit.

Where the test for content uniformity on granules is required, the test for weight variation may not be carried out.

Filling quantity For multidose granules, comply with the test for minimum fill (Appendix V F).

Microbial limit Comply with the microbial limit test (Appendix XIII G), unless otherwise specified. Where the sterility test on granules is required, the microbial limit test may not be carried out.

I K Powders

Powders are the preparations made of dry powders of drug substances of biologics and suitable subsidiary materials by the process of pulverization and homogenization.

Powders for oral administration, which generally dissolve and disperse in water or other liquids, can be administered directly with water.

The production and storage of powders shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.
2. The ingredient for preparing powders shall be dried and made into fine powders. Powders shall be dry, loose, mixed well and uniform in appearance and colour.
3. Powders may contain or not contain subsidiary materials. Flavoring agents, aromatics or colouring agents may be added during preparation of powders for oral administration if necessary.

4. To prevent the destruction of active ingredients in powders by gastric acid, the ingredients with capacity of neutralizing gastric acid may be added to the diluent of powders.

5. Powders are packaged as single-dose or multi-dose preparations, and a dose dividing tool shall be attached for multidose preparations.

6. Powders containing volatile or hygroscopic medicaments shall be packaged with moisture-proof materials.

7. Unless otherwise specified, powders shall be stored and shipped at 2-8°C in well-closed containers.

Powders shall be subject to the following tests.

Fineness Weigh accurately 10 g of test sample, unless otherwise specified, place on sieve No. 7 (125 μ m), cover the sieve with a lid, fit tightly an appropriate receiver under the sieve. Carry out the test for particle size (Appendix V G). Weigh accurately the fractions passing through the sieve. The weight shall be not less than 95% of the powder weighed.

Uniformity of appearance Spread evenly a quantity of powders in an area of about 5 cm² on a piece of smooth paper, press the surface to be even, observe the powder under a bright light. It shall be uniform in colouration without discolourations or colour stains.

Loss on drying Carry out the test for loss on drying (Appendix VII L), unless otherwise specified. Losses shall be not more than 2.0% of its weight after drying to constant weight at 105°C.

Weight variation The weight variation of single-dose powders shall comply with the following requirements.

Average or labelled weight	Weight variation limit
0.1 g or less than 0.1 g	$\pm 15\%$
More than 0.1 g up to 0.3 g	$\pm 10\%$
More than 0.3 g up to 1.5 g	$\pm 7.5\%$
More than 1.5 g up to 6.0 g	$\pm 5\%$
More than 6.0 g	$\pm 3\%$

Procedure Take 10 packs (bottles) of test sample, remove the wrapper, and accurately weigh the content of each separately. Calculate the weight of each pack (bottle) and the average weight. Compare the weight of each pack (bottle) with the average weight (for the granules without content determination, compare the weight of each pack (bottle) with the labelled weight). Not more than 2 individual values shall exceed the limit, and none shall exceed by doubling the limit. Where the test for content uniformity for powders is required, the test for weight variation may not be carried out.

Filling quantity The multidose powders shall

comply with the test for minimum fill (Appendix V F).

Microbial limit Comply with the microbial limit test (Appendix XIII G), unless otherwise specified. Where the test for contaminating microorganisms on powders is required, the microbial limit test may not be carried out.

I L Nasal Preparations

Nasal preparations are liquid, semisolid or solid preparations, intended for administration to the nasal cavities to produce a systemic or local therapeutic effect. The dose forms of liquid preparations include nasal drops, washings and sprays, for semisolid preparations including ointments, emulsion and gel, and for solid preparations including powders, powder aerosols and club suppositories.

The production and storage of nasal preparations shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.

2. In general, nasal preparations contain the subsidiary materials which have the capacity of adjusting viscosity, controlling pH, enhancing the dissolution of active ingredients, or improving the stability of preparations, or may be used as excipients. Other than the preparations with the capacity of bacteriostasis, bacteriostatic agents at suitable concentrations shall be added to aqueous nasal preparations supplied in multidose containers, unless otherwise specified.

3. The containers of multidose nasal preparations shall be fitted with a set of dropping pipettes, generally with spiral caps and rubber or plastic nipples. The containers shall be innocuous and clean, and shall not react physically or chemically with medicaments or subsidiary materials. The walls of containers shall be in a certain and even thickness. Unless otherwise specified, the filling quantity in each container shall be not more than 10 ml or 5 g.

4. Liquid nasal preparations shall be clear and free from precipitates or foreign matters. Nasal suspension may contain precipitates which shall be easily dispersed on shaking. For nasal emulsion phase separation between oil and water may occur which can be restored on shaking.

5. Nasal preparations shall be nonirritating and shall not adversely affect the function of nasal mucosa and its cilia. Liquid nasal preparations shall be isotonic.

6. Unless otherwise specified, nasal preparations shall comply with the corresponding requirements

in the General Requirements for Preparations. For example, nasal sprays shall comply with the requirements for sprays.

Unless otherwise specified, nasal preparations shall be stored and shipped at 2-8°C, protected from light.

Nasal preparations shall be subject to the following tests.

Variation of weight or of filling quantity Unless otherwise specified, the variation of weight or of filling quantity of solid or semisolid nasal preparations supplied in single-dose containers shall comply with the following requirements.

Procedure Take 20 containers of test sample, weigh the content of each container and calculate the average weight. Not more than 2 containers shall deviate from the average weight by $\pm 10\%$, and none shall deviate by $\pm 20\%$.

Where the test for content uniformity of nasal preparations is required, the tests for variation of weight or of filling quantity may not be carried out.

Filling quantity Unless otherwise specified, single-dose nasal preparations shall comply with the following requirements.

Take 10 containers of test sample, remove the total contents and determine the filling quantity of each container, which shall be not less than the labelled quantity.

Multidose nasal preparations shall comply with the test for minimum fill (Appendix V F).

Microbial limit Comply with the microbial limit test (Appendix XIII G), unless otherwise specified. Where the sterility test on nasal preparations is required, the microbial limit test may not be carried out.

Sterility The sterile nasal preparations as stated on the label shall comply with the test for sterility (Appendix XIII A).

I M Gels

Gels are homogeneous suspended or emulsive thick liquid or semi-solid preparations, which are made of drug substances, in liquid or dry powder form, and suitable gelling subsidiary materials. Unless otherwise specified, gels are intended for local application to skin, nasal cavity, vagina and rectum. Emulsive liquid gels are also called as emulsive gels. A gel of small molecule of inorganic medicaments (e.g. aluminum hydroxide) consists of a network of small discrete gel particles of drug substances presenting in liquid. It is classified as a two-phase dispersion system, and is also called as

suspended gel which is thixotropic. It is in a semi-solid form in stationary and changes into a liquid by stirring or shaking.

The bases of hydrophilic gels usually consist of water, glycerin or propylene glycol gelled with suitable gelling agents such as tragacanth, gelatin, starch, cellulose derivatives, carbomer and alginates. The bases of hydrophobic gels consist of liquid paraffin with polyethylene glycol or fatty oil gelled with colloidal silica or aluminium soap or zinc soap.

The production and storage of gels shall comply with the following requirements.

1. The drug substances used shall comply with the requirements in relevant monographs.

2. Colloid particles in suspended gels shall disperse homogeneously. No sedimentation or caking shall occur.

3. Gels shall be homogeneous, fine and kept in gel structure without drying up or liquefaction at room temperature.

4. Additives such as moisturizers, preservatives, antioxidants, emulsifying agents, thickeners and transdermal accelerators may be added to gels if necessary.

5. The bases of gels shall not interact physically or chemically with medicaments.

6. "Shake up before use" shall be stated on the label of suspended gels.

7. The gels used for severely injured skin, nasal cavity or vagina shall be sterile.

8. The immediate packaging materials for gels shall not interact physically or chemically with medicaments or bases.

9. Unless otherwise specified, gels shall be preserved in well-closed containers and stored and shipped at 2-8°C, protected from light or freezing. Gels shall be subject to the following tests.

Particle size Unless otherwise specified, spread a quantity of the suspended gels onto three microscope slides separately with a thin layer whose area is equivalent to the covered slide. Carry out the test for particle size (Appendix V G, method 1), and no particle of greater than 180 μm in dimension shall be observed.

Filling quantity Comply with the test for minimum fill (Appendix V F).

Microbial limit Comply with the microbial limit test (Appendix XIII G), unless otherwise specified. Where the sterility test on gels is prescribed, the microbial limit test may not be carried out.

Sterility Sterile gels shall comply with the test for sterility (Appendix XIII A).

Appendix II Spectrophotometry

Spectrophotometry is a method used in qualitative and quantitative analysis in which the light absorption or the intensity of light emission of the substance being examined is measured at a definite wavelength or within a definite range of wavelength.

The spectral ranges involved in pharmaceutical analysis mainly consist of 3 regions; (1) the ultraviolet region (200-400 nm), (2) the visible region (400-760 nm) and (3) the infrared region (2.5-25 μm or 4000-400 cm^{-1}). The instruments used are the ultraviolet-visible spectrophotometer, visible spectrophotometer (or colourimeter), infrared spectrophotometer or atomic absorption spectrophotometer. All instruments shall be calibrated regularly to insure the precision and the accuracy.

When monochromatic radiation passes through an absorbing medium, the absorbance of the radiation is proportional to the concentration of the absorbing substance and the thickness of the absorbing medium, this relation is expressed by the following equation:

$$A = \lg 1/T = ECL$$

Where A is the absorbance, T is the transmittance, E is the absorption coefficient, C is the concentration of the substance expressed in g per 100 ml, calculated on the dried or dehydrated basis and L is the absorption path length expressed in cm. The term $E_{1\%}^{1\text{cm}}$ is used in this pharmacopoeia to denote the absorbance of a 1% solution in a 1 cm cell.

The wavelength of the selective absorption and the corresponding absorption coefficient are physical constants of the substance being examined. When the absorption coefficient of a substance is known, its content can be calculated from the above equation. In the visible region, the content of a colourless substance can be determined colourimetrically after the addition of a colour developing agent or any other treatment.

II A Ultraviolet-visible Spectrophotometry

Calibration and performance test of the instrument

1. Wavelength The change of environment may affect the mechanical parts of the spectropho-

tometer and cause a drift of the wavelength scale, therefore it must be calibrated regularly and immediately before the measurement. Mercury lamp is the best choice of light source for this purpose, the following spectral lines of the mercury lamp can be used; 237.83 nm, 253.65 nm, 275.28 nm, 296.73 nm, 313.16 nm, 334.15 nm, 365.02 nm, 404.66 nm, 435.83 nm, 546.07 nm and 576.96 nm. The wavelength scale may also be calibrated by means of the 486.02 nm and 656.10 nm lines of deuterium discharge lamp. Holmium glass filter exhibits sharp absorption peaks at 279.4 nm, 287.5 nm, 333.7 nm, 360.9 nm, 418.5 nm, 460.0 nm, 484.5 nm, 536.2 nm and 637.5 nm therefore can be used for calibration of wavelength. However, the exact values for the position of these peaks may change slightly depending on the commercial source of the filter or along with the time goes by.

2. The absorbance scale. Check the absorbance with a solution of potassium dichromate in sulfuric acid. Dissolve about 60 mg of potassium dichromate primary standard, previously dried to constant weight at 120°C and accurately weighed, in 0.005 mol/L sulfuric acid solution to make 1000 ml. Check the absorbance at wavelength indicated in the following table and calculate the absorption coefficient which shall accord with the specified range in the table when compared with the specified absorption coefficient.

Wavelength/nm	235(min)	257(max)	313(min)	350(max)
Specific absorbance $E_{1\%}^{1\text{cm}}$	124.5	144.0	48.6	106.6
Maximum tolerance	123.0-126.0	142.8-146.2	47.0-50.3	105.5-108.5

3. Limit of stray light. Stray light may be detected at the given wavelength with suitable solutions indicated in the following table. The transmittance of these solutions measured in a 1 cm quartz cell against water shall accord with the limit specified in the table.

Reagent	Concentration/% (g/ml)	Wavelength/nm	Transmittance/%
Sodium Iodide	1.00	220	<0.8
Sodium Nitrite	5.00	340	<0.8

Requirements for the solvents

The organic solvents containing heteroatoms

usually have strong absorption at the lower wavelength. Thus, their ranges of use shall be less than the cut off wavelength. For example, the cut off wavelength of methanol and ethanol is 205 nm. Otherwise, impurity of solvents would enhance the interference of absorption. The solvent used in spectrophotometric determinations should be checked for any interfering absorption peak around the selected wavelength for the measurement of the absorbance being examined. The absorbance of a solvent shall not exceed 0.40 in the range of 220 nm to 240 nm, 0.20 in the range of 241 nm to 250 nm, 0.10 in the range of 251 nm to 300 nm and 0.05 at wavelengths above 300 nm, when measured in a 1 cm quartz cell against air.

Procedure

Unless otherwise specified, the same batch of solvent used to prepare the solution of the substance being examined should be employed as the blank in matched 1 cm quartz cells. The wavelength of maximum absorption shall be checked by measuring the absorbance of the substance being examined in the vicinity of the specified wavelength, within a range of ± 2 nm, to check the wavelength of the absorption maximum is correct or not. Unless otherwise specified, the absorption maximum must be within ± 2 nm as specified in the monograph, otherwise, the identity, purity of the substance or the correctness of the wavelength of the spectrophotometer should be considered. The assay should be carried out at the wavelength of maximum absorption. The concentration of the solution shall be adjusted to give an absorbance reading of 0.3 to 0.7 where the experimental error is the smallest. The width of the spectral slit must be smaller than the tenth of half-width of the absorption band, otherwise low absorbance will be resulted. The slit width is appropriate if further reduction does not result in an increase of the absorbance reading. The absorbance of an unmatched cell with the solvent concerned as a blank must be subtracted from the absorbance of the substance being examined or be automatically deducted by the spectrophotometer.

When the pH value affects the results determined, the pH of the test preparation shall be adjusted equal to that of the reference preparation.

Ultraviolet spectrophotometry used for identification and quality tests is proceeded according to the method described in the items specified under the corresponding monograph.

Ultraviolet spectrophotometry is used for assay usually as the following methods.

(1) Reference substance comparison method

Prepare separately solutions of the substance being examined and CRS according to the method described under item of the individual monograph. The content of the CRS in the solution shall be within $100\% \pm 10\%$ of the labelled amount of the solution prepared with substance being examined

and with solvent of the same batch. Determine the absorbances of solutions of test preparation and of reference preparation at specified wavelength, calculate the concentration of the test preparation in the solution according to the following equation:

$$C_x = (A_x/A_R)C_R$$

Where C_x is the concentration of the test preparation; A_x is the absorbance of the test preparation; C_R is the concentration of the reference preparation and A_R is the absorbance of the reference preparation.

(2) *Absorption coefficient method* Prepare the solutions of the substance being examined according to the method described under the individual monograph, determine the absorbance at specified wavelength. Calculate the concentration of the test preparation with the absorption coefficient specified in the monograph concerned. Usually, absorption coefficient shall be more than 100. Pay attention to the calibration and check of the instrument being used.

(3) *Chemometric methods* Carry out the assay as described under the specified monograph. Absorbances are measured at wavelengths of the ascending or descending position of the absorption curve, the minor variation of the wavelength may affect the result significantly. It is essential to determine the reference and test preparations under the same condition. Commonly, chemometric method is not suitable for the assay.

(4) *Colourimetry* Colourimetry is used with the addition of suitable developer before determination when the substance being examined has no strong absorption in the ultraviolet-visible region, or though absorption in that region, to avoid the interference or increase the sensitivity.

Colourimetric determination shall be carried out with a reference substance concomitantly. Unless otherwise specified, an equal volume of solvent, added with the same reagent and treated in the same manner is used as blank. Calculate the concentration of test preparation as "(1)Reference substance comparison method" described under the above method (1).

If the linear relation between absorbance and concentration is not good enough, the absorbance of a series of reference preparations containing gradient amounts of the reference substance shall be measured, and a calibration curve shall be produced by plotting the absorbance against concentration. The concentration of the test preparation can be determined by interpolating its absorbance on the calibration curve.

II B Atomic Absorption Spectrophotometry

Atomic absorption spectrophotometry is used in

the determination of metal elements and some non-metal elements in the atomic state.

The light of characteristic wavelength emitted from a cathodic discharge lamp is absorbed when it passes through the atomic vapor generated from sample containing the element being examined atomized to the ground state. The assay of the element being examined is tested by determining the decreased degree of light intensity of radiation. Atomic absorption obeys the general rule for absorption spectrophotometry. The assay is carried out by comparing the absorbance of the test preparation with that of the reference preparation.

Apparatus

An atomic absorption spectrophotometer consists of a light source, an atomic generator, a monochromator and a detector system. Some are equipped with a background compensation system and automatic sampling system, etc.

1. Light source A hollow-cathode discharge lamp is usually used. The cathode is made of the element being examined.

2. Atomic generator There are four main types: flame atomizer, graphite furnace atomizer, hydride-generated atomizer and cold vapor atomizer.

(1) *Flame atomizer* It mainly consists of a nebulizer and a burner. Its function is to nebulize the test solution into aerosol which is mixed with combustion gas. And the mixture is introduced into the flame generated by the burner. So that the substance being examined is to be dried, evaporated to form the ground state atoms of the element being examined. The burning flame is generated by different mixtures of gases, acetylene-air is mostly used. By modifying the proportion of combustion gas, the temperature of the flame can be controlled, and a better stability and a better sensitivity can be obtained.

(2) *Furnace atomizer* It consists of electric furnace and a power supply. Its function is to dry and incinerate the substance being examined. During the stage of high temperature atomization, the ground state atoms of the element being examined are to be formed. Graphite is commonly used as the heater. Protection gas is introduced into the furnace to avoid oxidation and used to transfer the sample vapor.

(3) *Hydride-generated atomizer* It consists of hydride generator and atomic absorption cell. It is used for the determination of the elements such as arsenic, selenium, stannum and antimony etc. Its function is to reduce the element to be examined in acidic medium to the low-boiling and easily pyrolyzed hydride. And then the hydride is swept by a stream of carrier gas into the atomic absorption cell which consists of quartz tube and heater etc., in which the hydride is pyrolyzed by heating to form the ground-state atom.

(4) *Cold vapor atomizer* It consists of a mercury vapor atomizer and an absorption cell. It is suitable for the determination of mercury. Its function is to reduce the mercuric ion into mercury vapor which is swept into the quartz absorption cell by carrier gas.

3. Monochromator Its function is to separate the specified wavelength radiation from the electromagnetic radiations radiated from the light source. The optical path of the apparatus should assure the good spectra resolution and has the ability to work well at the condition of narrow spectral band (0.2 nm). The commonly used wavelength region is 190.0-900.0 nm.

4. Detector system It consists of a detector, a signal processor and a recording system. It should have relatively higher sensitivity and better stability, and can follow the rapid change of the signal absorption.

5. Background compensation system System employed for the correction of atmospheric effects on the measuring system. Four principles can be utilized for background compensation: continuous spectrum sources (a deuterium lamp is often used in the UV region), the Zeeman effect, the self-inversion phenomena and the nonresonance spectrum. In the analysis using atomic absorption spectrophotometry, the interference to the determination caused by background and other reasons should be noticed. Changes of some experimental conditions, such as the wavelength, the slit width, the atomizing condition, etc., may affect the sensitivity, the stability and the interference. If it is flame, the suitable wavelength, slit width and flame temperature, the addition of complexing agents and releasing agents, and the use of Standard addition method may eliminate interference. If it is furnace, system, the selection of suitable background compensation system and the addition of suitable matrix modifying agents, etc. may remove the interference. Background compensation method shall be selected as specified in the individual monograph.

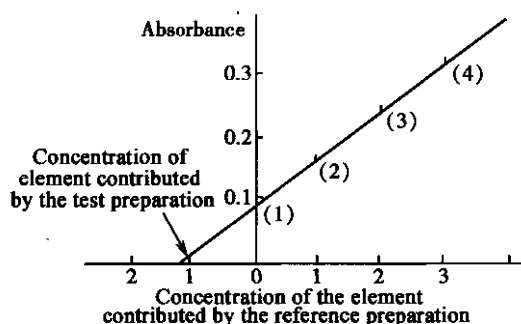
Procedure

Method 1 (Direct calibration method) Prepare not less than 3 reference solutions of the element being examined of different concentrations, covering the range recommended by the instrument manufacturer and add separately the corresponding reagents as that for the test solution and prepare the blank reference solution with the corresponding reagents. Measure the absorbances of the blank reference solution and each reference solution of different concentrations separately, record the readings and prepare a calibration curve with the average value of 3 readings of each concentration on the ordinate and the corresponding concentration on the abscissa.

Prepare a test solution of the substance being

examined as specified in the monograph, adjust the concentration to fall within the concentration range of the reference solution. Measure the absorbance 3 times, record the readings and calculate the average value. Interpolate the mean value of the readings on the calibration curve to determine the concentration of the element.

Method 2 (Standard addition method) Place equal volumes of the test preparation prepared as specified in the individual monograph in each of 4 volumetric flasks of the same size, to each of the flasks, except the first one, add an accurately measured amount of the reference preparation containing increasing amounts of the element being determined. Dilute separately with de-ionized water to the volume and proceed as directed in the direct calibration method. Measure the absorbances and record the readings. Plot the mean values of each group of 3 readings against the corresponding concentration of the element contributed by the reference preparation, extrapolate the straight line to intersect with the axis of zero absorbance. The interception represents the concentration of the element contributed by the test preparation. Calculate the concentration of the element in the test preparation from the result so obtained. This method can be used only when the standard curve obtained in method 1 is linear and passes the origin.



When used in the test for impurities, prepare two test preparations of the same concentration as specified in the monograph. To one of the test preparation add an amount of the reference substance equivalent to the limit of the element specified in the monograph. Proceed as directed above and measure this solution to give an appropriate reading a ; then measure the test preparation without the addition of the reference substance under the same condition and record the reading b ; b is not greater than $(a-b)$.

II C Fluorimetry

When substances are exposed to ultraviolet or visible radiation, some of them may emit fluorescence at a wavelength longer than that of

the exciting radiation. The excitation and emission spectra of a substance can be used for qualitative analysis. The intensity of the fluorescence emitted by a substance, when the intensity and wavelength of the exciting radiation, the solvent and the temperature are constant, is directly proportional to the concentration of the substance within a definite range, therefore, this relation can be used for quantitative determinations. The sensitivity of fluorescence spectrophotometry is usually higher than that of UV and visible spectrophotometry. However, if a solution is too concentrated, a self-quenching effect and an absorption of the exciting radiation near the surface may result in a declination of the intensity of emitted radiation, and the intensity of fluorescence emitted is not directly proportional to the concentration. Therefore, fluorescence spectrophotometry should be carried out only in dilute solutions.

Procedure The instrument used is fluorometer or fluorometry spectrophotometer. Select the excitation and emission bands and prepare the reference and the test solutions as specified in the monograph. Measurements are usually made with reference to a properly selected reference substance to determine the linear relation of fluorescence intensity and concentration. When the linear relation is good, adjust the sensitivity of the instrument with appropriate dilutions of the reference solution before each test; record the fluorescence intensities of the test preparation and reference preparation and their corresponding blanks. The following equation can be used for calculation of the content in the test preparation:

$$C_x = \frac{R_x - R_{xb}}{R_r - R_{rb}} \times C_r$$

Where C_x is the concentration of the test preparation; C_r is the concentration of the reference preparation; R_x is the fluorescence intensity of the test preparation; R_r is the fluorescence intensity of the reference preparation; R_{xb} and R_{rb} are the fluorescence intensity of the corresponding blanks.

The range within which the fluorescence intensity is directly proportional to the concentration of the substance is usually very narrow, therefore, the ratio $(R_x - R_{xb}) / (R_r - R_{rb})$ shall be not less than 0.5 or more than 2, otherwise the concentration of the solutions shall be adjusted and the measurements made again. If the fluorescence intensity is not strictly proportional to the concentration, the previously drawn calibration curve under the same conditions shall be used.

For a substance which decomposes on exposure to light or its relaxation time is too long, in order to avoid the fluorescence intensity affected by multi-irradiation of the exciting radiation, the sensitivity of the instrument maybe checked with a stable reference solution of another fluorescent substance with excitation and emission bands similar to those

of the substance being examined in place of reference substance of the substance being examined. For instance, quinine in dilute sulfuric acid is usually used for blue fluorescence, sodium fluorescence for green fluorescence and rhodamine B for red fluorescence.

Announcements The interferences of fluorometry are great due to high sensitivity.

- (1) The purity of solvent may markedly affect the intensity of fluorescence, blank test should be carried out and the solvent distilled in a glass distillator before use if necessary.
- (2) The presence of suspended particles may cause the light to be scattered, therefore, it is necessary to eliminate such particles by centrifugation or filtration with a sintered glass filter.
- (3) All glasswares and cells must be cleaned thoroughly.
- (4) It is also important to regulate the temperature because it would notably affect the fluorescence intensity.
- (5) Oxygen dissolved in the solution has a strong quenching effect, it can be removed by passing a current of inert gas through the solution when necessary.
- (6) The effect on the fluorescence intensity by the pH value of the solution and the purity of the reagents, etc. should be noticed during the determination.

alkali metals and alkaline-earth metals being examined is introduced into a flame as the form of aerosol by an equipment of nebulization, the element being examined is atomised by the heat energy of the flame and excited its characteristic spectrum. The content of the element being examined is determined by measuring the light intensity of the element using photoelectric detector and comparing the light intensity of the reference solution and the test solution.

Apparatus Flame photometer consists of a combustion system including nebulizer-burner, combustion lamp, combustion gas and supply of assisted combustion gas, a monochromator and a detector. The combustion gas is often a mixture of air-coal gas or air-liquefied petroleum gas (LPG) using air as assisted-combustion gas and coal gas or LPG as combustion gas.

Any variation of the experimental condition, such as type and state of flame, the pressure supplied by air-compressor may affect and interfere with the sensitivity and steadiness of the instrument, so they should be selected as specified in the monograph.

Procedure When used for assay and limit test of impurities, flame photometry is carried out respectively as method 1 and 2 described under Atomic Absorption Spectrophotometry (Appendix II B).

II D Flame Photometry

When a test solution containing the element of

Appendix III Chromatography

Depending on the mechanism of the separation process, chromatographic can be classified into adsorption chromatography, partition chromatography, ion-exchange chromatography, size-exclusion chromatography etc.

Adsorption chromatography is based on the different affinity of individual components to the adsorbent (stationary phase), so that they can be eluted successively with a solvent or gas (mobile phase). The adsorbents commonly used are aluminum oxide, silica gel, polyamide powder etc.

Partition chromatography is based on the distribution of individual components between two phases. The stationary phase is coated on or chemically bonded to a solid support of large surface area. The mobile phase is a liquid or a gas. The supports commonly used are silica gel, kieselguhr, diatomaceous earth, cellulose powder, polymers with suitable functional groups etc.

In ion-exchange chromatography, the stationary phase is either a cation exchange resin or an anion exchange resin. The mobile phase is usually an aqueous buffer solution, sometimes a definite quantity of organic solvent is added to modify the exchange property.

Size exclusion chromatography is also known as gel permeation chromatography or gel filtration chromatography. It is based on the different permeability of components of different molecular size into a support of definite pore size. Molecular sieves, cross-linked polystyrene gels, glucosan gels, silica gel and porous glass beads are commonly used as the stationary phase. Water or organic solvent is used as the mobile phase, depending on the chemical characteristics of the support and the substance being examined.

Chromatography may be classified into paper chromatography, thin layer chromatography, column chromatography, gas chromatography, high performance liquid chromatography and so on based on the method of separation.

Solvents used in chromatography must be of high purity and must not react with the substance being examined. Except gas chromatography, the operation is carried out at room temperature, unless otherwise specified. In column chromatography, paper chromatography and thin layer chromatography, the coloured zones can be detected

visually, colourless substances can be detected under ultraviolet radiation of 254 nm or 365 nm. In paper chromatography or thin layer chromatography, colourless substances can also be detected by spraying with a colour-developing agent. Silica gel plates containing a fluorescent substance are sometimes used in thin-layer chromatography, so that colourless substance can be detected by the fluorescence quenching method. In column chromatography, gas chromatography and high performance liquid chromatography, the components can be detected by a suitable detector connected to the outlet of the column. In column chromatography, the components sometimes can be determined quantitatively by a suitable method after fractionation.

III A Paper Chromatography

Paper chromatography is a partition chromatographic technique, in which paper is used as a support and the water or other substance contained in the paper is used as a stationary phase.

The substance being examined is developed by the developer, after which the shift ratio value (R_f) could be used to denote the location of each component. (R_f is the ratio of the distance between the center of the origin and that of the spots to the distance between the center of the origin and the frontal of the mobile phase). In practice, R_f values may vary considerably due to experimental conditions. Therefore, the identification of a compound is usually carried out by comparing the behaviour of the compound to be identified with that of a reference substance under the same conditions. For drug identification, the location and colour (or fluorescence) of the main spots of the substance being examined on the chromatogram shall be identical with those of the reference substances. For purity inspection, the number or colour intensity (or fluorescence intensity) of the impurities contained shall be examined according to the requirements specified under each monograph, after a certain amount of substance being examined has been developed. For assay, the main chromatographic spot could be cut out from the paper, eluted with a solvent and subjected to a suitable measurement.

1. Apparatus and Materials

(1) *Developing chamber* It is usually a glass chamber, cylindrical or rectangular, which could be tightly closed with a glass lid. When it is used for descending method, a separator could be inserted through the lid with a hole on it for the addition of the mobile phase. A solvent trough supported by a rack is placed inside near the top of the chamber, a glass rod is used for holding the chromatographic paper, on both sides of the trough, there are two glass rods used to guide the paper so that no part of it is in contact with the wall of the trough. When it is used for ascending method, the hole on the lid is blocked by a stopper with a glass hook where the paper is suspended. The hook is capable of being lowered without opening the chamber. Remove the trough and rack.

(2) *Sample applicator* Microsyringe or capillary tubes with a rack are often used, which could ensure a correct and compact position of the applied spot.

(3) *Chromatographic filter paper* Filter paper should be clean and uniform in texture and thickness, and has a tensile strength. It shall contain no impurities which may affect developing process or react with visualizing reagent employed, and affect the separation or identification. It may be pretreated before use if necessary. For descending method, cut a piece of filter paper along the grain direction into strips of sufficient length and a convenient width, and draw a fine pencil line horizontally across the paper at such a distance from one end to make the line a few centimeters below the guide rod. When this end is secured in the solvent trough and the remainder of the paper is hanging freely outside the trough. The lower end of the paper may be cut into saw-toothed form to facilitate the mobile phase to drop down homogeneously, if necessary. For ascending method, the length of the paper is about 25 cm, the width of the paper is varied as required. Sometimes the paper can be rolled into cylindrical form to save space, the spotting line is about 2.5 cm from the lower edge of the paper.

2. Procedure

(1) *Descending method* Dissolve the substance being examined in a suitable solvent or solvent mixture to prepare a solution of specified concentration. Apply the solution in portions to the pencil line, allow it to dry in air or in a stream of warm air before the next application. The spots are commonly 2-4 mm in diameter, and spaced about 1.5-2.0 cm apart from each other in circular shape. Place the spotted end of paper in the solvent trough and hang the paper freely over the guide rod. Put a Petri dish containing the specified solvent or a strip of chromatographic filter paper moistened with the specified solvent into the chromatographic chamber to pre-equilibrate the chamber with the vapour of the specified solvent.

Introduce a sufficient quantity of the mobile phase into the solvent trough and allow it to move along the chromatographic paper for the prescribed distance. Remove the chromatographic paper from the chromatographic chamber, mark the position of the solvent frontal and visualize the chromatogram prescribed in the monograph when the solvent is volatilized.

(2) *Ascending method* The solution of substance being examined is applied on the pencil line as directed in the descending method. The chamber is saturated with the vapour of a specified solvent or the developer placed in it. Lower the hook so that the paper is immersed in the developer to a depth of 0.5 cm, allow the mobile phase to ascend for about 15 cm, unless specified otherwise. Remove the paper from the chamber, mark the position of the solvent frontal and visualize the chromatogram as prescribed in the monograph when the solvent is volatilized.

One dimensional chromatography is that the development proceeds along one direction. Sometimes two-dimensional chromatography may be performed by turning the filter paper at right angle and then continue the development with the original developer or a different solvent system. Other chromatographic techniques, such as multiple development, continuous development and wedge shaped strip development etc., may also be used.

III B High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a method of chromatographic separation in which the mobile phase is pumped into a column containing stationary phase by a high-pressure pump system. The test solution injected is carried into the column by the mobile phase. All the components are separated in the column and pass through the detector sequentially. The recorder, integrator or data acquisition system thus records the chromatographic signals.

1. General requirements for the instrument

The HPLC instrument is used here, which should be checked periodically and meets the requirements of relative specification.

(1) *Chromatographic column* The most widely used packing material is the chemically bonded silica gel. Reverse-phase chromatographic system uses nonpolar packing materials, in which the most frequently used is octadecylsilane bonded silica gel. Octylsilane and other type of chemically bonded silica gel (such as cyano and amino group bonded silica gel) are also used. Positive-phase chromatographic system uses polar packing

material, the most widely used of which is silica gel. The ion-exchange resin is used in ion-exchange chromatography. The gel and macromolecular porous microsphere are used in size exclusion chromatography. Chiral bonded packing materials are used in separation of enantiomers (chiral chromatography).

The characteristics of the packing materials (the shape of the support, the particle size, the pore diameter, the surface area, the surface coverage of the bonded functional group, the carbon content and the bonding mode, etc.) and the pack of the chromatographic column effect directly the retention behavior and separation performance. The packing material with pore diameter less than 15 nm (1 nm=10 Å) is suitable for analysis of the compound whose molecular weight is less than 2000, pore diameter more than 30 nm is suitable for analysis of compound whose molecular weight is more than 2000.

The mobile phase in which the pH value is 2-8 is suitable for the stationary phase with silica gel as the support. If the pH value is more than 8, the silica gel may be dissolved and if the pH value is less than 2, the phase chemically bonded with the silica gel may be broken off. In the case when the mobile phase in which the pH value is more than 8 should be used, the packing material, which is alkali-proof, should be chosen, such as bonded silica gel with high purity silicon as support and a high surface coverage, polymer coated packing material, the organic and inorganic hybridized packing materials and the non-silica gel packing materials. In the case when the mobile phase in which the pH value is less than 2 should be used, the packing material, which is acid-proof, may be chosen. Such as the octadecylsilane modified silica gel with diisopropyl or diisobutyl substitution, which has a bulk mass and thus results in a protection through a steric hindrance, or organic and inorganic hybridized packing material, etc.

(2) *Detectors* The most commonly used detector is the ultraviolet (UV) spectrophotometers. Besides, diode array detectors (DAD), fluorescence spectrophotometers, differential refractometers, evaporative light scattering detectors, electrochemical detectors and mass spectrometers may also be used.

The UV spectrophotometers, diode array detectors, fluorescence spectrophotometers and electrochemical detectors are all selective detectors. The response value is related not only to the concentration of the test solution, but also to the structure of the compound. On the contrary, the differential refractometers and evaporative light scattering detectors are general-purpose detectors; they will respond to all compounds. The response value of the evaporative light scattering detectors for compounds with similar structure is almost related to the mass of the test compound. Diode array detector may record the absorption spectrum

within the prescribed wavelength range simultaneously, thus could be used to measure the spectrum and inspect the purity of the chromatographic peaks.

The response values of the UV spectrophotometers, fluorescence spectrophotometers, electrochemical detectors and differential refractometers are linear with the concentration of the test solution within a certain range, while the response value of the evaporative light scattering detectors is not always linear with the concentration of the test solution, if necessary, it should be transferred mathematically before calculation.

Different detectors have different requirements for the mobile phase. For example, if the UV spectrophotometer is used in the experiment, the mobile phase should at least meet the requirements for the solvent described in the UV spectrophotometry (Appendix II A). When the light of lower wavelength is applied for detection, the cut-off wavelength of the organic solvent should be considered and it is better to use chromatographic grade organic solvent. The evaporative light scattering detectors and the mass spectrometers generally do not allow using the mobile phase that contains non-volatile salts.

(3) *Mobile phase* As the C₁₈ chain is not able to keep an spread state in the water solution, the ratio of the organic solvent of the mobile phase shall not less than 5%, in the reverse phase chromatographic system of which the stationary phase is octadecylsilane bonded silica gel. Otherwise the random curl of the C₁₈ chain will lead to the change of the retention value of the component, which may result in the instability of the chromatographic system.

The type of the stationary phase, the composition of the mobile phase and the mode of the detectors specified under the monograph shall not be changed. Others, such as the inner diameter, the length, the brand of the stationary phase and the particle size of the support, the flow rate of the mobile phase and the ratio of each component in the mixed mobile phase, the time span in the gradient elution program, the temperature of the column, the injection volume and the sensitivity of the detectors could be changed appropriately to meet the requirements of the system suitability test. But for some certain monographs, in which only the specific brand of packing material is able to satisfy the requirements of the separation, clear indications should be given under them.

2. The system suitability test

The suitability test of the chromatographic system generally includes four indexes, number of theoretical plates, resolution, repeatability and tailing factors, in which the resolution and the repeatability are more practical indexes.

To carry out the suitability test of the chromatographic system according to the requirements under

the individual monograph is to test the chromatographic system by using specific reference substance. The separation conditions of the chromatography should be adjusted in case if the requirement cannot be met.

(1) *Theoretical plates of the column (n)* Inject the test solution or the internal standard specified under the monograph into the system according to the prescribed chromatographic conditions. Record the chromatogram and measure the retention time t_R (in minutes or length units, the same as below, which should be in the same unit) and the peak width at half peak height ($W_{h/2}$) of the principal component peak of the test solution or that of the internal standard. Calculate the theoretical plates of the chromatographic column by the equation: $n = 5.54(t_R/W_{h/2})^2$.

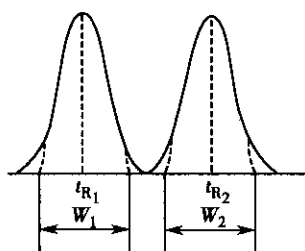
(2) *Resolution (R)* No matter in qualitative analysis or quantitative analysis, the peak (s) of substance being examined and other peaks, such as internal standard peak (s) or specific impurity peaks must have good resolution. The equation for calculating the resolution is:

$$R = \frac{2(t_{R2} - t_{R1})}{W_1 + W_2}$$

Where t_{R2} is the retention time of the latter of the two adjacent peaks, t_{R1} is the retention time of the former of the two adjacent peaks.

W_1 and W_2 are the peak width of the two adjacent peaks.

Unless specified otherwise, the resolution value shall be not less than 1.5 in quantitative analysis.



(3) *Repeatability* Inject the reference solution described under the monograph for 5 times successively. Unless specified otherwise, the relative standard deviation of the measured value of the peak areas shall be no more than 2.0 per cent.

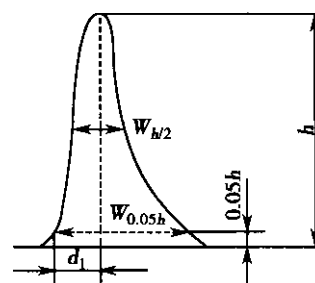
According to the measurement of the correction factor specified under each monograph, prepare a series of reference solutions equivalent to 80%, 100% and 120% of the content of the substance being examined, each containing a definite amount of internal standard substance, inject each solution at least 2 times, calculate the average correction factors, the relative standard deviation of which shall be no more than 2.0%.

(4) *Tailing factor (T)* In order to guarantee

the performance of the separation and the precision of the measurement, the tailing factor should be inspected to see whether it meets the prescription under each monograph. The equation for calculating the tailing factor is as follows:

$$T = \frac{W_{0.05h}}{2d_1}$$

Where $W_{0.05h}$ is the peak width at 5% of the peak height, d_1 is the distance between the perpendicular line passing through the peak maximum and that of the leading edge of the peak.



Unless otherwise specified, the T value shall be within 0.95-1.05 in the quantitative analysis by using peak height. In the quantitative analysis by using peak area, the over deviation of the T value will also effect the detection and the quantification precision of the small peak (s).

3. Procedure

(1) *Corrected internal standard method for the determination of individual impurities or the main components*

Prepare solutions containing an accurately weighed quantity of the reference substance and the internal standard respectively as specified in the monograph. Accurately measure each solution and prepare the reference solution for determining the correction factors. Inject a certain amount of solution into the equipment and record the chromatogram. Measure the peak area or height of the reference substance and the internal standard and calculate the correction factor according to the following equation:

$$\text{Correction factor}(f) = \frac{A_S/C_S}{A_R/C_R}$$

Where A_S is the peak area or the peak height of the internal standard

A_R is the peak area or the peak height of the reference substance

C_S is the concentration of the internal standard

C_R is the concentration of the reference solution

Prepare solutions containing the substance being examined and the internal standard as described in the monograph. Inject the solution into the equipment and record the chromatogram. Measure the peak area or the peak height of the substance

being examined and that of the internal standard. Calculate the content as follows:

$$\text{Content}(C_x) = f \times \frac{A_x}{A'_s/C'_s}$$

Where A_x is the peak area or peak height of the substance being examined (or its impurity)

C_x is the concentration of the solution of the substance being examined (or its impurity)

A'_s is the peak area or the peak height of the internal standard solution

C'_s is the concentration of the internal standard solution

f is the correction factor.

If an equal amount of the internal standard of the same concentration is used both in preparing the reference solution for measuring the correction factor and in preparing the test solution, that is $C_s = C'_s$. In this case accurate weighing is not necessary for preparing the internal standard solution.

(2) External standard method for the determination of the individual impurity or the main component

Prepare solutions containing an accurately weighed quantity of the reference substance and the substance being examined respectively and inject certain amount of each solution into the equipment. Record the chromatogram and measure the peak area (or peak height). Calculate the content as follows:

$$\text{Content}(C_x) = C_R \frac{A_x}{A_R}$$

Where each symbol has the same meaning as that mentioned above.

As the microsyringe is not able to control the amount of solution being injected precisely, the sampling loop or automatic sampler may be used in measuring the content of the impurity and main components in the substance being examined.

(3) Corrected peak areas of impurities compared with that produced by the main peak of a diluted solution of substance being examined

This method could be used to measure the content of the impurity. Prepare solutions containing an accurately weighed quantity of the reference substances of impurities and the main component as specified in the monograph. Inject a volume and record the chromatogram. Calculate the correction factor of the impurity according to the method described in the (1). The correction factor could be directly recorded in the individual monograph and used for correction of the measured peak areas of impurities. These impurities, which need correction computation, are generally located by the relative retention time to the main component as the reference and all these data are recorded in the individual monograph.

When measuring the content of the impurities, dilute the solution of the substance being examined to a concentration as specified in the individual monograph so that its peak area is around that produced by the impurities in the original concentration. Inject and adjust the attenuation of the detector (limited by the noise level that can be accepted) or vary the injection volume (limited by the load ability of the column) until the peak height of the main component is about 10%-25% of the full scale or the peak area which could be accurately measured (generally, for the impurity, the content of which is lower than 0.5%, the RSD of the peak area shall be less than 10%; while for the impurity, the content of which is between 0.5%-2%, the RSD of peak area shall be less than 5%; for the impurity whose content is higher than 2%, the RSD of the peak area shall be less than 2%). Inject separately an appropriately amount of test solution and reference solution. The recorded time span of the test solution, unless specified otherwise, shall be 2 times that of the main component. Measure the peak areas of each impurity on the chromatogram of the test solution. Multiply them by the respective correction factors and then compare them with the peak area of the main component of the reference solution and calculate the content of the impurities accordingly.

(4) Peak areas of impurities compared with that produced by the main peak of a diluted solution of substance being examined

This method could be used when the impurity reference substance is not available. Prepare the reference solution according to method (3) and adjust the attenuation of the detector. Inject separately an appropriate volume of the test solution and reference solution. The record time span of the former shall be 2 times the retention time of the main component, unless specified otherwise. Measure the peak areas of the impurities on the chromatogram of the test solution and compare them with those of the main component of the reference solution and calculate the content of the impurities.

If there are some impurities in the substance being examined which are not completely separated from the solvent peak, record the chromatogram I of the substance being examined according to the specification and then record the chromatogram II of the pure solvent of equal volume. The peak area of the solvent solution on the chromatogram II is subtracted from the total peak area of the impurities in the chromatogram I (including the solvent peak). The result is equal to the correction peak area of the total impurities. Calculate the content of the impurities accordingly.

(5) Peak area normalization method

As the deviation of this method is so large that it can be only used to roughly inspect the contents of impurities in the substance being examined. It is not suitable for the determination of minute

impurities, unless specified otherwise. In this method, the peak area of the impurities and the total peak area on the chromatogram except the solvent peak are measured. Calculate the peak area of each impurity and the percentage of their sum in the total peak area.

III C Gas Chromatography

Gas chromatography (GC) is a separation technique in which the mobile phase, an inert gas known as carrier gas, is passing through the chromatographic column packed with packing materials for separation and determination. The substance or its derivatives are injected into the vaporizer with a micro-syringe and vaporized, separated on the stationary phase, each component passes through the detector in succession and a chromatogram is thus recorded by integrator, recorder or data acquisition system.

1. General requirements for the instrument

The apparatus consists of a carrier gas source, an injector port, a chromatographic column contained in an oven, a detector and a data acquisition system. The injection port, column, and detector are temperature-controlled.

(1) **Carrier gas source** The mobile phase of gas chromatography is gas, known as carrier gas. Carrier gases used are usually nitrogen, helium and hydrogen. The gas is supplied by a high-pressure steel cylinder or high-purity gas generator and passes through suitable pressure-reducing valves and a flow meter to the injector port and column. The gas selection depends on the properties of the substances being examined and the species of the detector. The commonly used carrier gas is nitrogen, unless specified otherwise.

(2) **Injection port** Direct injections of solutions and headspace injection are the usual modes of injection.

Direct injection may be carried out by using a syringe or an injection valve, or into a vaporization chamber which may be equipped with a stream splitter. The temperature of the vaporizer is usually 30-50°C higher than that of the column when using direct injection. The volume of solution injected is not more than several μl . The smaller the column diameter the less volume of injected solution is. Capillary column is used with injectors which is able to split samples into two fractions to avoid overloading.

Headspace injectors are suitable for separation and determination of volatile component in solid or liquid substance being examined. The test solutions produced with solid or liquid substance being examined and stored in tightly closed containers are heated in the chamber for a period of

time, allowing the volatile components in the test solution to reach a equilibrium between the nongaseous phase and the gaseous phase. A predetermined amount of the head-space of the vial is flushed into the column by automatic syringe.

(3) **The chromatographic column** The chromatographic columns are classified into the packed columns and capillary columns. The packed columns, which are made of stainless steel or glass, are 2-4 mm in internal diameter and 2-4 m in length. The columns are packed with the sorbent, porous polymer bead or the supports coated with liquid phase, particle size of which is 0.25-0.18 mm, 0.18-0.15 mm or 0.15-0.125 mm. The support usually used is acid washed and silanized diatomaceous earth or porous polymer beads; the liquid phase commonly used is methylpolysiloxane, polysilphenylene of different consist, polyethyleneglycol etc. The capillary columns are made of glass or quartz. The inner wall of the column is coated or cross-linked with stationary liquid, the internal diameter is usually 0.25 mm, 0.32 mm or 0.53 mm, the length is 5-60 m, and the film thickness of the stationary liquid is 0.1-5.0 μm . The stationary liquid commonly used are methyl polysiloxane, phenylmethylpolysiloxane with different ratio of composition and carbowax, etc.

New packed and capillary column must be conditioned before use to remove oxygen and residual solvents. The chromatographic column must be conditioned before use until the baseline is stable if the column is not in use long time.

(4) **The column oven** The temperature-controlled precision of the oven shall be $\pm 1^\circ\text{C}$ and fluctuation of temperature shall be less than 0.1°C per hour, as the temperature fluctuation of oven will influence the reproducibility of chromatographic analysis result. Temperature-controlled system may be classified into constant temperature and temperature programming.

(5) **Detector** The detectors suitable for the gas chromatography include flameionization detector (FID), thermal conductivity detector (TCD), nitrogen-phosphorus detector (NPD), flame-photometer detector (FPD), electron-capture detector (ECD), mass spectrometric detector (MSD) etc.. FID responses well to hydrocarbon and is suitable for the determination of most drug compounds; NPD is sensitive to organic nitrogen and phosphorus compounds; FPD is sensitive to organic sulfur and phosphorus compounds; ECD is suitable for determination of halogen compounds; MSD can offer chemical construction information of the compounds which is useful for structure verification. Unless specified otherwise, the detector should be FID which employs hydrogen as combustion gas and air as combustion-supporting gas. When FID is used, its temperature is higher

than that of the column and not less than 150°C to prevent condensation of the vapour, which usually is 250-350°C.

(6) **Data process system** It is classified into recorder, integrator and computer station etc. If necessary, parameters which specified under the individual monograph, except the kind of the detector, the type of the stationary phase and the material of the column, other parameters such as the internal diameter and length of the column, brand and size of the support, concentration of the stationary phase, the flow rate of carrier gas, the temperature of the column, the injection volume, the sensitivity of the detector etc, may be varied to meet the requirement of the system suitability test. Usually the chromatogram is completed within 30 minutes.

2. System suitability test

The requirements are the same as described under High Performance Liquid Chromatography, unless otherwise specified.

3. Procedure

(1) Corrected internal standard method for the determination of individual impurities or the main component.

(2) External standard method for the determination of individual impurities or the main component.

(3) Peak area normalized method.

The specific content of (1)-(3) is the same as described under High Performance Liquid Chromatography.

(4) Standard addition method for the determination of individual impurities or the main component. Dissolved an accurately weighed quantity of impurities or reference substance of the substance to be examined to produce reference solution with a suitable concentration. Measure accurately the solution and add to the test solution, then calculate the content of individual impurities or the main component by internal standard method or external standard method. Deduct the content of added standard solution and gain the content of individual impurities or the main component in the test solution.

The content may also be calculated by the following equation. The correction factor is the same as that of adding the reference solution.

$$\frac{A_{is}}{A_x} = \frac{C_x + \Delta C_x}{C_x}$$

The concentration C_x of component being examined may be calculated by the following equation.

$$C_x = \frac{\Delta C_x}{(A_{is}/A_x) - 1}$$

Where C_x is the concentration of component X being examined;

A_x is the peak area of component X being examined;

ΔC_x is the concentration of added reference

substance of component being examined.
 A_{is} is the peak area of component X after adding reference substance of component being examined.

In quantitative assay of gas chromatography, a major source of error is the irreproducibility in the amount of sample injected, which is affected by retaining time of syringe and room temperature, notably when manual injections are made with a syringe. The effects of variability can be minimized by an internal standard. Automatic injectors greatly improve the reproducibility of sample injections and reduce the need for internal standards. When headspace injectors are equipped, the effect of matrix may be eliminated by standard addition method because the test solution and reference solution are in different matrix. When the quantitative result of standard addition method is different from others, the result of standard addition method should be adopted.

III D Size Exclusion Chromatography

Size-exclusion chromatography is a liquid chromatographic technique that separates molecules in solution according to their size. The separation of size-exclusion chromatography is based on molecule sieve mechanism of the column. Hydrophilic silica gel, gel or modified gel, such as sephadex and sepherose, etc., are usually used as the packing material of the column. The different size pores are distributed in the surface of the packing material. When the sample is introduced into the column, the molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute earliest by the mobile phase, their retention time is the shortest. The molecules which are smaller than all pores size penetrate all the pore spaces and elute latest, their retention time is the longest. Other molecules elute through the column in sequence according to their molecule size.

1. General requirements for the apparatus

The injector and detector are as same as those for High performance Liquid Chromatography. Pumping systems typically includes normal pressure pump, middle pressure pump and high pressure pump. High performance size-exclusion chromatography (HPSEC) is the most frequently adopted in pharmaceutical analysis, especially in determination of molecular weight and molecular weight distribution. The packing material adopted should be suitable for the molecular weight of samples. Mobile phase is usually aqueous solution or buffer solution, pH value of which should not exceed endurance of the packing material and is

better between 2 and 8. A suitable quantity of organic modifier can be added in the mobile phase, but the concentration should not be high and usually not exceed 30%. Flow rate should not be fast and is usually 0.5-1.0 ml/min.

2. System suitability test

Generally the determination method of the number of theoretical plates (n), resolution, repeatability and tailing factor of the column for system suitability test is as same as the method stated in High Performance Liquid Chromatography. But if monomer and dimer of some drug molecule cannot be separated by baseline during determination of macromolecule impurities, resolution is calculated by the following formula:

$$R = \frac{\text{peak height of dimer}}{\text{valley height between monomer and dimer}}$$

Resolution is more than 2.0, unless otherwise specified in the monograph.

3. Procedure

(1) *Determination of molecular weight* The method is generally suitable for determination of molecular weight of protein and peptide. Carry out the method stated in the monograph, using a column and reference substances suitable for the molecular weight of samples. Reference substance and samples should both be processed by dithiothreitol (DTT) and sodium lauryl sulfate (SDS) in order to break disulfide bond and make the molecule configuration and conformation concord. Usually the processed protein and peptides which have been turned to a straight line are separated. Plot a graph of the retention time of the reference substance as a function of the logarithm molecular weight and calculate the equation of linear regression, $\lg M_w = a + bt_R$. Calculate molecular weight or subunit molecular weight from the regression equation

(2) *Determination of molecular weight and molecular weight distribution of polymers*

Molecular weight of biopolymers, such as amylose, nucleic acid and collagen, is usually unhomogeneous. Molecular weight and molecular weight distribution of the biopolymer is a key index. To determine the molecular weight and molecular weight distribution of biopolymers, it is important to adopt reference substances with similar structures and properties to the samples. Carry out the method stated in the monograph, unless otherwise specified, use molecular weight reference substance and suitable GPC software, plot a graph of the retention time of the reference substance as a function of the logarithm of weight-average molecular weight and calculate the equation of linear regression $\lg M_w = a + bt_R$. Process the result with suitable GPC software and calculate the molecular weight and molecular weight distribution of samples.

$$M_n = \sum RI_i / \sum (RI_i / M_i)$$

$$M_w = \sum (RI_i / M_i) / \sum RI_i$$

$$D = M_w / M_n$$

Where M_n is number-average molecular weight
 M_w is weight-average molecular weight
 D is distribution coefficient
 RI_i is peak height of samples at retention time i
 M_i is molecular weight of samples at retention time i

(3) Determination of macromolecule impurities

Macromolecule impurities are the impurities with higher molecular weight than drug molecule, which produced during procedure of manufacture or storage and cannot be removed completely (sensitizing polymers).

Separation is carried out according to the chromatographic condition stated in the monograph.

Quantitative method

① *Peak areas of impurities compared with those produced by the main peak of the sample* See the section of High Performance Liquid Chromatogram. The method is typically used for determination of macromolecule impurities with low content in the samples.

② *Peak area normalization method* See the section of High Performance Liquid Chromatogram.

③ *Retention time limit method* Unless otherwise specified, it is specified that component with shorter retention time than that of the main peak is not allowed to be detected. This method is typically used to control macromolecule impurities in the mixture.

④ *External standard method by the substance to be examined* This method is typically used for determination of macromolecule impurities in β -lactam antibiotics by Sephadex G-10 gel chromatography system. Except for some oligomers, macromolecule impurities in β -lactam antibiotics are not retained in this system and only appear to be one peak. Calculate content of the macromolecule impurities by external standard method, using the substance to be examined as reference substance.

Announcements Processing method for Sephadex G-10

Packing chromatographic column Soak about 15 g of Sephadex G-10 with water for 48 hours before packing and make it swelling thoroughly, stir to expel air bubbles. Slowly add the mixture as slurry into the chromatographic tube. Wash down the Sephadex G-10 adhering to the inner wall of the tube with water, smooth the surface of the column. Newly packed column should be eluted with water for 4-6 hours to expel air bubbles.

Loading the sample Both automatic inject valve and manual loading can be adopted to load the substance being examined into the column. Add the test solution slowly along the inner wall of the

tube, care should be taken that the packing material is not disturbed. Wash down the samples adhering to the inner wall of the tube with 3-5 ml

mobile phase after sample solution permeate through the column surface.

Appendix IV Electrophoresis

Electrophoresis is a method employing an inert supporting medium (such as paper, cellulose acetate, agarose gel, and polyacrylamide gel, etc.), in which the electrically charged test samples (protein and nucleic acid, etc.) migrate towards the electrode of opposite charge under the action of an electric field. Each component migrates at its own speed and is separated in narrow zone. The electrophoretogram and content of every component can be examined with appropriate assaying methods. Various electrophoreses are carried out according to the following procedures unless otherwise specified.

IV A Cellulose Acetate Film Electrophoresis

Reagent

(1) Barbitol buffer solution (pH 8.6)
Dissolve 2.76 g of barbitol and 15.45 g of sodium barbitol in water and dilute to 1000 ml.

(2) Amino black staining solution
Dissolve 0.5 g of amino black 10B in a mixture of 50 ml of methanol, 10 ml of glacial acetic acid and 40 ml of water.

(3) Washing solution
Mix 45 ml of ethanol, 5 ml of glacial acetic acid and 50 ml of water thoroughly.

(4) Hyalinization solution
Mix 25 ml of glacial acetic acid and 75 ml of absolute ethanol thoroughly.

Procedure

Cut a piece of cellulose acetate film into strips with a dimension of 2 cm × 8 cm. Immerse the strips in barbitol buffer solution (pH 8.6) with their rough side downward. After soaking thoroughly, take out the strips and absorb the excess buffer solution carefully with filter paper. Place the strips on the electrophoresis frame with their rough side upward. Immerse the film into barbitol buffer solution (pH 8.6) through filter paper Bridge. Load 2 - 3 μ l of test sample (containing about 5% protein) dropwise along a line of the strip 2 cm from the cathode edge. Connect and control the current at 0.4 - 0.6 mA/cm [total current = current (mA/cm) × width of each strip (cm) × number of strips] At the same time, a control test is carried out using fresh human

serum. Continue the electrophoresis until the distance between albumin and gamma globulin is about 2 cm.

After electrophoresis, immerse the strips in amino black staining solution for 2 - 3 minutes. Then, wash with washing solution repeatedly until the background is colourless. Immerse the washed and completely dried strips in the hyalinization solution until fully soaked. Take out the strips and spread flatly on a clean glass plate. Transparent films are obtained after drying. The films can be used for purity determination and be kept as specimen for long-term storage.

Scan the dry cellulose acetate film with chromatographic scanner by means of reflection (for non-transparent film) or transmission (for transparent film). A curve of protein fractions is plotted with an automatic recorder. The percentage of each protein fraction (%) is calculated according to their peak areas using human serum as a control.

IV B Agarose Electrophoresis

Reagent

(1) Barbitol buffer solution (pH 8.6)
Dissolve 4.14 g of barbitol and 23.18 g of sodium barbitol in a volume of water by heating. Cool down to room temperature and add 0.15 g of sodium azide. After sodium azide is dissolved, dilute the solution to 1500 ml with water.

(2) 1.5% agarose solution
Add 50 ml of water and 50 ml of barbitol buffer solution (pH 8.6) to 1.5 g of agarose. Heat the mixture until the agarose is completely swollen.

(3) 0.5% Amino black solution
Dissolve 0.5 g of amino black 10B in a mixture of 50 ml of methanol, 10 ml of glacial acetic acid and 40 ml of water.

(4) Destaining solution
Mix 45 ml of ethanol, 5 ml of glacial acetic acid and 50 ml of water thoroughly.

(5) Bromophenol blue indicator solution
Dissolve 50 mg of bromophenol blue in water and dilute to 100 ml.

Reference substance

Normal human serum or other suitable reference substances can be used.

Preparation of test sample solution

Dilute the test sample to a protein concentration of 1%-2% with physiological saline.

Procedure

Pour the hot 1.5% agarose solution onto a horizontal glass plate of suitable size to a thickness of 3 mm. Allow to stand to form a thin and even agarose gel plate which is free of bubble. Punch wells at the one-third length of the agarose gel plate apart from the cathode. The diameter of each well is 2-3 mm. Put the plate onto an electrophoresis trough containing barbital buffer solution (pH 8.6). Add a quantity of test sample solution and one drop of bromophenol blue indicator solution into test sample well. Add a quantity of reference substances and one drop of bromophenol blue indicator solution into the control well. Connect the two ends of the gel plate to barbital buffer solution (pH 8.6) through three layers of filter paper. Perform electrophoresis for 2 hours at a constant voltage of 100 V until the indicator migrates to the forward position. Stain the plate with 0.5% amino black solution after electrophoresis and then decolourize in destaining solution until the background is colourless.

IV C SDS-Polyacrylamide Gel Electrophoresis

Most proteins may form complex with sodium dodecyl sulfate (SDS), an anionic surfactant, and make the negative charges on protein complexes much more than that on the natural proteins. The charge-effect of different proteins is then eliminated and proteins are separated according to their molecular sizes.

Instrument

Power source supplying constant voltage or constant current shall be used. Disc or vertical plate electrophoretic troughs and gel preparation mould shall be used.

Reagent**(1) Water**

The specific electric resistance of water used shall be not less than $18.2 \text{ M}\Omega \cdot \text{cm}$.

(2) Solution A 1.5 mol/L Tris (hydroxymethyl) aminomethane hydrochloric acid solution

Dissolve 18.15 g of Tris (hydroxymethyl) aminomethane in a quantity of water. Adjust pH to 8.8 with hydrochloric acid and dilute to 100 ml with water.

(3) Solution B

30% acrylamide-0.8% N,N'-methylene-bisacrylamide (protected from light)

(4) Solution C

1% sodium dodecyl sulfate (SDS)

(5) Solution D

10% tetramethylethylenediamine

(6) Solution E

10% ammonium persulfate

(7) Solution F

0.5 mol/L tris-(hydroxymethyl)-aminomethane-hydrochloric acid solution

Dissolve 6.05 g of tris-(hydroxymethyl)-aminomethane in a quantity of water. Adjust pH to 6.8 with hydrochloric acid and dilute to 1000 ml with water.

(8) Electrode buffer solution

Dissolve 3 g of tris-(hydroxymethyl)-aminomethane, 14.4 g of glycine and 1 g of sodium dodecyl sulfate in a quantity of water. Adjust pH to 8.3 with hydrochloric acid and dilute to 1000 ml with water.

(9) Test sample buffer solution

Dissolve 0.303 g of tris-(hydroxymethyl)-aminomethane, 2 mg of bromophenol blue, 0.8 g of sodium dodecyl sulfate, 0.189 ml of hydrochloric acid and 4 ml of glycerol in water and dilute to 10 ml. This solution is used for non-reducing SDS-PAGE. For reducing SDS-PAGE, add 2 ml of β -mercaptoethanol into the solution.

(10) Molecular weight standard

The molecular weight of test sample shall be in the range of molecular weight of protein standards used.

(11) Fixing solution

Dilute 250 ml of methanol and 60 ml of glacial acetic acid to 500 ml with water.

(12) Washing solution

Dilute 100 ml of ethanol and 50 ml of glacial acetic acid to 1000 ml with water.

(13) Auxiliary staining solution

Dissolve 10 g of potassium dichromate in a quantity of water. Add 2 ml of nitric acid and dilute to 200 ml with water. Make 40-fold dilution before use.

(14) Silver staining solution

Dissolve 2.04 g of silver nitrate in water and dilute to 1000 ml.

(15) Developing solution

Dissolve 30 g of sodium carbonate in a quantity of water. Add 0.5 ml of formalin and dilute to 1000 ml with water.

(16) Stopping solution

Dilute 10 ml of glacial acetic acid to 1000 ml with water.

(17) Coomassie brilliant blue staining solution

Dissolve 1 g of Coomassie brilliant blue R-250 in a mixture of 200 ml of methanol, 50 ml of glacial acetic acid and 250 ml of water.

(18) Coomassie brilliant blue destaining solution

Mix 400 ml of methanol, 100 ml of glacial acetic acid and 500 ml of water thoroughly.

Preparation of test sample solution

Mix three volumes of test sample with one volume of test sample buffer solution thoroughly. Heat in 100°C water bath for 3 - 5 minutes.

Procedure**(1) Preparation of separating gel**

Prepare the separating gel according to the

compositions listed in the following table and inject the gel into the electrophoresis trough to a certain height. Cover the top with water and polymerize at room temperature. The polymerization time is changeable at different room temperatures.

Type of gel	Separating Gel						Stacking Gel
Gel concentration	5%	7.5%	10%	12.5%	15%	17.5%	4.5%
Solution A(ml)	4	4	4	4	4	4	
Solution B(ml)	2.7	4	5.4	6.7	8	9.4	1.35
Solution C(ml)	1.6	1.6	1.6	1.6	1.6	1.6	0.9
Solution D(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.07
Solution E(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.07
Solution F(ml)							2.25
Water(ml)	7.3	6	4.88	3.3	2.28	0.88	4.33

(2) Preparation of stacking gel

After the separating gel is polymerized, blot out the water on its top with filter paper and inject in stacking gel (see formula in the above table). Insert the test sample comb carefully to prevent the occurrence of air bubbles.

(3) Sample loading

Remove the sampling comb after the stacking gel is polymerized. Fill both front and rear electrophoretic troughs with electrode buffer solution. Add 5 μ g (intended for silver staining) or more than 10 μ g (intended for Coomassie brilliant blue staining) of the treated test sample into the sample loading well.

(4) Electrophoresis

Connect circuit and turn on condensed water. Perform electrophoresis at a constant current of 10 mA until the test sample enters the separating gel. Then, adjust the current to 20 mA until electrophoresis is completed.

(5) Fixing and Staining

① Silver staining method

Silver staining method is not used for quantitative determination unless otherwise specified. The amount of test sample loaded may be properly increased for qualitative tests.

Immerse the gel after electrophoresis into fixing solution overnight. Take out the gel and wash 3 times, 10 minutes for each time, with washing solution at temperatures not lower than 25°C.

After washing, immerse the gel into auxiliary staining solution for 7-10 minutes. Take out the gel and wash 3 times by immersing into water, 2 minutes for each time.

After washing, immerse the gel into silver staining solution. Expose to relatively strong sunlight or similar light source for 30 minutes, allow to stand under indoor light for 20 minutes. Take out the gel from silver staining solution and wash twice by immersing in water, 1 minute for each time.

After washing, immerse the gel into developing solution. Change the solution every 2 minutes until all the protein bands develop completely.

Immerse the gel into stopping solution for 10 minutes and then preserve in water.

② Coomassie brilliant blue staining method

Immerse the gel after electrophoresis into Coomassie brilliant blue staining solution overnight. Take out the gel from staining solution and immerse into destaining solution until the background is almost colourless. Take out the gel and preserve in water.

Result calculation

Scan the non-reducing electrophoresis gel for purity analysis. Scan the reducing electrophoresis gel to obtain molecular weight standard curve and calculate the molecular weight of test sample.

IV D Isoelectric Focusing Electrophoresis

Ampholytes form a pH gradient in an electrophoretic field. Protein, as an ampholyte, carries the electric charges related to the pH value of the medium. The charged protein will migrate towards the electrode of opposite charge during electrophoresis and stop at the position with a minimum migration force where the pH value is equal to its isoelectric point (at this point, the specified protein no longer carries electric charge). This method is used for the determination of the isoelectric point of protein test samples.

Method 1 Vertical gel isoelectric focusing

Reagent

(1) Water

The specific electric resistance of water shall be not less than 18.2 M Ω · cm.

(2) Solution A

Dissolve 29.1 g of acrylamide and 0.9 g of N, N'-methylene-bisacrylamide in a quantity of water and dilute to 100 ml. Filter with two layers of filter paper and store protected from light.

(3) Solution B

A 10% ammonium persulfate solution is prepared just before use.

(4) Test sample buffer solution (4 times of concentration)

Dilute 8 ml of glycerin and 4 ml of 40% ampholytes solution (pH 3 - 10) with water to 20 ml. Add 20 μ l of 0.1% methyl red solution.

(5) Fixing solution

Dissolve 34.5 g of trichloroacetic acid and 10.4 g of sulfosalicylic acid in water and dilute to 300 ml.

(6) Destaining solution (Balancing solution)

Dilute 500 ml of 95% ethanol and 160 ml of glacial acetic acid with water to 2000 ml.

(7) Staining solution

To 0.35 g of Coomassie brilliant blue G250 (or R250), add 300 ml of destaining solution. Heat in 60 - 70°C water bath until the solid is dissolved.

(8) Preserving solution

Mix 30 ml of glycerin and 300 ml of destaining solution thoroughly.

(9) Cathode solution (0.01 mol/L phosphoric acid solution)

Dilute 1 ml of phosphoric acid to 1800 ml with water.

(10) Anode solution (0.01 mol/L sodium hydroxide solution)

Dissolve 0.4 g of sodium hydroxide with a small volume of water and dilute to 1000 ml with water.

Preparation of test sample solution

Desalt the test sample by dialyzing against water, or by other methods, and mix with test sample buffer solution at a volume ratio of 3 : 1. The final concentration of the test sample solution shall be more than 0.5 mg per ml. If the concentration is too low, the test sample shall be concentrated by a suitable method.

Procedure

Set up the vertical electrophoresis trough and squeeze out water. Add 1 ml of 60% glycerin between cellophane paper and glass plate. Mix 12 ml of water, 2 ml of glycerin, 4.0 ml of solution A and 1.0 ml of ampholyte (pH 3-10) solution (or other ampholytes) thoroughly. Mix well and degas. Add 72 μ l of solution B and 3 μ l of N, N, N', N'-tetramethyl-ethylenediamine (TEMED), and mix well. Inject the mixture into the trough and polymerize for one hour. Add 20 μ l of test sample buffer solution to each well. Connect cooling water and perform electrophoresis at 10°C for 30 minutes under 250 V (about 10 mA). Load 20 μ l of test sample to each well. Perform the electrophoresis at 10°C for about 3.5 hours under 500 V (about 10 mA) with a maximum of 2000 V. A control test is performed at the same time using isoelectric focusing markers instead of test sample. After electrophoresis, fix the gel for more than 20 minutes in fixing solution and transfer to balancing solution for 20-30 minutes. Then, immerse the gel into staining solution for 40-60 minutes. Decolourize the gel with destaining solution until the background is colourless and transfer to preserving solution for 30 minutes. The gel may also be preserved as a dry film.

A linear regression equation is obtained by regressing the pI values of the isoelectric focusing markers with the corresponding migration distances. The isoelectric point of test sample is obtained by inserting the migration distance of test sample into the equation.

Method 2 Slab gel isoelectric focusing

Reagent

(1) Water

The specific electric resistance of water shall be not less than 18 M Ω · cm.

(2) Solution A

Dissolve 29.1 g of acrylamide and 0.9 g of N, N'-methylene-bisacrylamide in a quantity of water and dilute to 100 ml. Filter with two layers of filter

paper and store protected from light.

(3) Solution B

A 10% ammonium persulfate solution is prepared just before use.

(4) Fixing solution

Dissolve 34.5 g of trichloroacetic acid and 10.4 g of sulfsalicylic acid in water and dilute to 300 ml.

(5) Destaining solution (Balancing solution)

Mix 500 ml of 95% ethanol and 160 ml of glacial acetic acid with water and dilute to 2000 ml.

(6) Staining solution

To 0.35 g of Coomassie brilliant blue G250 (or R250), add 300 ml of destaining solution. Heat in 60 - 70°C water bath until it is dissolved.

(7) Preserving solution

Mix 30 ml of glycerin with 300 ml of destaining solution thoroughly.

(8) Cathode solution (0.5 mol/L phosphoric acid solution)

Dilute 50 ml of 85% phosphoric acid to 1800 ml with water.

(9) Anode solution (0.2 mol/L sodium hydroxide solution)

Dissolve 8 g of sodium hydroxide in water and dilute to 1000 ml.

Preparation of test sample solution

Desalt the test sample by dialyzing against water or by other methods. Adjust the protein concentration of test sample to more than 0.5 mg per ml. If the concentration is too low, the test sample shall be concentrated by a suitable method.

Procedure

The polyacrylamide gel solution is prepared by mixing 6.25 ml of solution A and 1.5 ml of pH 3-10 ampholyte (or other ampholyte) with 17.1 ml of water. Degas by suction for 5-10 minutes. Then, add 175 μ l of solution B and 20 μ l of N, N, N', N'-tetramethylethylenediamine (TEMED). Mix well. Inject the gel solution slowly into a horizontal mould and polymerize at room temperature. Smear liquid paraffin or kerosene on the surface of the cooling plate and put on the prepared polyacrylamide gel carefully to prevent the occurrence of air bubbles. Moisten the cathode strip with cathode solution and the anode strip with anode solution, and then put them onto the cathode and anode respectively. Place filter paper for sample loading on the gel. Load 5-30 μ l of test sample solution. Lay the electrodes at the center of electrode strips and lid a cover. Perform the electrophoresis for 2.5 hours at 4°C at a voltage with an upper limit of 2000 V and a current with an upper limit of 50 mA. The power is 1 W per cm of gel. A control test is performed at the same time using isoelectric focusing markers instead of test sample. Remove the filter paper for sample loading after the first 30 minutes of electrophoresis. After electrophoresis, fix the gel in fixing solution for more than 20 minutes and transfer to balancing solution for 20-30 minutes. Stain the gel in staining solution for 40-60 minutes and then wash by immersing into

destaining solution until the background is colourless. Take out the gel and put into preserving solution for 30 minutes. Dry the gel in air. The gel may also be prepared into a dry film for long-term storage.

A linear regression equation is obtained by

regressing the pI values of the isoelectric focusing markers with the correspondent migration distances. The isoelectric point of test sample is obtained by inserting the migration distance of test sample into the equation.

Appendix V

V A Determination of pH Value

The pH value of an aqueous solution is determined by a pH meter using a glass electrode as the indicator electrode, and a saturated calomel electrode as the reference electrode. Metrological verification of the pH meter should be carried out at regular intervals to meet the related national requirements. Before each measurement, the pH meter should be calibrated with the standard buffer solutions prepared as follows, or those of a declared pH value accurate to 0.01 pH unit distributed by national administrative department of certified reference material (CRM).

1. Standard buffer solutions used for the calibration of pH meters are prepared as follows.

(1) *Standard tetraoxalate BS* Dissolve 12.71 g of potassium tetraoxalate, previously dried at $54^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 4-5 hours and accurately weighed, in water to produce 1000 ml.

(2) *Standard biphthalate BS* Dissolve 10.21 g of potassium biphthalate, previously dried at $115^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 2-3 hours and accurately weighed, in water to produce 1000 ml.

(3) *Standard phosphate BS* Dissolve 3.55 g of anhydrous disodium hydrogen phosphate and 3.40 g of potassium dihydrogen phosphate, previously dried at $115^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 2-3 hours and accurately weighed, in water to produce 1000 ml.

(4) *Standard sodium tetraborate BS* Dissolve 3.81 g of sodium tetraborate, accurately weighed (avoid efflorescence), in water to produce 1000 ml. Preserve the solution in well closed polyethylene containers, protected from carbon dioxide in the air.

(5) *Standard calcium hydroxide BS* Use the supernatant of a saturated solution of calcium hydroxide, saturated with carbon dioxide-free water at 25°C . Store protected from carbon dioxide in the air. Discard and repeat the preparation if the solution becomes turbid.

Standard buffer solutions mentioned above must be prepared from the certified reagents for pH determination. The exact pH values of the standard buffer solutions at different temperatures

mentioned above are given in the following table.

Temp. °C	Standard buffer solutions ^①				
	1	2	3	4	5
0	1.67	4.01	6.98	9.64	13.43
5	1.67	4.00	6.95	9.40	13.21
10	1.67	4.00	6.92	9.33	13.00
15	1.67	4.00	6.90	9.28	12.81
20	1.68	4.00	6.88	9.23	12.63
25	1.68	4.01	6.86	9.18	12.45
30	1.68	4.02	6.85	9.14	12.29
35	1.69	4.02	6.84	9.10	12.13
40	1.69	4.04	6.84	9.07	11.98
45	1.70	4.05	6.83	9.04	11.84
50	1.71	4.06	6.83	9.01	11.71
55	1.72	4.08	6.83	8.99	11.57
60	1.72	4.09	6.84	8.96	11.45

① The standard buffer solutions are numbered here in the same sequence as shown in the preceding paragraph.

2. Announcements Operate the pH meter according to the manufacturer's instructions and pay attention to the following precautions when pH value is determined.

(1) Select two standard buffer solutions with difference in pH value of 3 units before determining test solution, and the pH value of the test solution is between that of two standard buffer solutions.

(2) Calibrate the apparatus using the primary standard buffer solution whose pH value is closer to test solution, adjusting the meter (fixed position) to read the appropriate pH value given in table mentioned above.

(3) Then calibrate the apparatus using the second standard buffer solution. The deviation shall be not more than ± 0.02 pH units, adjusting the slope carefully to make the observed pH value meet the value in the table mentioned above if it is not. Repeat the adjusting procedure for fixed position and slope until the difference between the observed value on the apparatus and the value of standard buffer solution is not more than 0.02 pH units. Otherwise the apparatus should be examined or the electrode should be exchanged till comply with the requirements.

(4) The electrode should be rinsed with water and dried (or rinsed with the solution being examined) before each measurement.

(5) A highly alkaline glass electrode should be

used if the pH value of the solution being examined is high. The error caused by alkalinity should be corrected if the electrode used is apt to produce such an error.

(6) The determination of the pH value of a liquid with weak buffering capacity (e.g. water) should be conducted after the pH meter is calibrated with standard potassium biphthalate BS and repeated after the pH meter is calibrated with standard sodium tetraborate BS. The readings should not be recorded until the shift in 1 minute is within 0.05 unit. The pH value of the liquid being examined is the mean value of the two readings provided that they do not differ by more than 0.1 unit.

(7) Freshly boiled and cooled distilled water with a pH value of 5.5-7.0 should be used for preparing standard buffer solutions and dissolving the substance to be examined in pH determinations.

(8) Usually, the standard buffer solutions can be kept for 2-3 months, but should not be used if any turbidity, mould or precipitate is discovered.

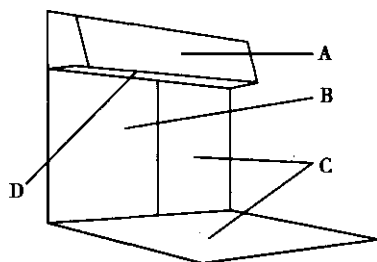
V B Test for Visible Particles (Lamp Test)

Visible particles refer to the insoluble substances that present in injections or eye drops and can be observed visually under specified conditions.

Introducing foreign particles during laboratory test shall be avoided. The operations of reconstituting freeze-dried preparations or transferring test sample to clean, transparent containers when the original containers are unsuitable for examination (for example, opaque, irregular in shape, etc.) shall be conducted in an environment with cleanliness of Class 100 (preferably in a laminar-air-flow cabinet).

Device for examination

The examination device is shown in the following diagram.



A indicates a fluorescent lamp fitted with a light baffle. The intensity of illumination may be adjustable in the range of 1000-3000 Lx.

B indicates the black background without light reflection.

C indicates the white background and bottom without light reflection (for examining coloured

foreign particles).

D indicates the white background with light reflection (i.e. inboard light baffle).

Requirements for inspector

By both near and distant vision tests, the visual acuity of inspectors shall be equal to or above 4.9 (the corrected visual acuity shall be equal to or above 5.0) and inspectors shall not have colour blindness.

Procedure

Injections Unless otherwise specified, take 20 containers of test sample, remove the labels from containers, clean the outer surfaces of containers, allow to stand at room temperature for a certain period of time (allow human albumin and human immunoglobulin to stand overnight in general). Carry out the visual examination in a light sheltered room or dark place. Hold the neck of containers at the edge of light baffle in an appropriate distance to inspector's eyes (usually 25 cm) and view against black and white backgrounds respectively by gently swirling and inverting the individual containers in order to make the visible particles, possibly existing in solutions, floating. (Care shall be taken to avoid the production of air bubbles.) The time limit for visual examination is 20 seconds. For the samples with a filling quantity of 10 ml or less than 10 ml, two containers shall be examined each time, and for the samples of more than 10 ml, one containers shall be examined each time. The injections with a filling quantity of 50 ml or more shall be examined by viewing vertically, horizontally and invertedly, respectively. The intensity of illumination for examining colourless solution shall be 1000-1500 Lx. For coloured solutions or the solutions in transparent plastic containers, the intensity of illumination for examination shall be 2000-3000 Lx.

Freeze-dried preparations for injection Unless otherwise specified, take 5 containers of test sample. Balance the temperatures of the test sample and the attached diluent to the temperature specified for reconstitution. Inject the diluent slowly along the wall of container and shake gently until the content is completely reconstituted. For test sample under vacuum, release the vacuum when the test sample is almost reconstituted and conduct the examination according to the above procedures; for test sample not under vacuum, conduct the examination according to the above procedures after the test sample is completely reconstituted.

Eye drops Unless otherwise specified, take 20 containers of test sample and examine according to the procedures for injections.

Result evaluation

Injections Unless otherwise specified, no glass scraps, fibers, colour dots, colour lumps or other visible foreign particles shall be found in all the 20 containers. Other visible particles found in

any containers shall be within the test limits as listed in the table below. The number of containers with visible particles beyond the test limits as listed in the table below shall be not more than one. If two containers are found with visible particles beyond the test limits, the test shall be repeated with additional 20 containers according to the same procedures. In both primary and repeat tests the total number of containers with visible particles beyond the test limits as listed in the table below shall be not more than two.

Freeze-dried preparations for injection Unless otherwise specified, no glass scraps, fibers, colour dots, colour lumps or other visible foreign particles shall be found in all the 5 containers. Other visible particles found in any containers shall be within the test limits as listed in the table below. If one container is found with visible particles beyond the test limits, the test shall be repeated with additional 10 containers according to the same procedures. In both primary and repeat tests the total number of containers with visible particles beyond the test limits as listed in the table below shall be not more than one.

Eye drops Unless otherwise specified, no glass scraps, fibers, colour dots, colour lumps or other visible foreign particles shall be found in all the 20 containers. Other visible particles found in any containers shall be within the test limits as listed in the table below. The number of containers with visible particles beyond the test limits as listed in the table below shall be not more than one. If two containers are found with visible particles beyond the test limits, the test shall be repeated with additional 20 containers according to the same procedures. In both primary and repeat tests the total number of containers with visible particles beyond the test limits as listed in the table below shall be not more than three.

Other types of visible particles	Filling quantity per container	Test limit per container
White dots, fine protein flocculus or protein particles	≤50 ml	≤3
	>50 ml	≤5
Small amount of flocculus or protein particles, a trace of precipitate or the precipitate not dispersed on shaking	/	Undetectable

The explanation of visible particles

- (1) While dots refer to the white particles without clear plane or edges.
- (2) Fine protein flocculus or protein particles refer to the translucent protein flocculus or protein particles of less than 1 mm in length.
- (3) Small amount of flocculus or protein particles refer to the protein flocculus or protein particles which are difficult to count within the specified time limit for the examination.
- (4) A trace of precipitate refers to a trace of precipitate formed after stationary standing,

which appears as a floating fog-like precipitate by gently inverting the container and disappears on gently shaking.

- (5) Precipitate not dispersed on shaking refers to a trace of precipitate formed in protein solutions after storage for a long time, which can not be dispersed on gently shaking.

[Note]

- (1) Sterility test shall be carried out on the samples with a loose cap or with a trace of precipitate.
- (2) Freeze-dried preparations shall be reconstituted under the specified temperature by the specified method for the said product.
- (3) Rubber scraps created by needling through rubber stoppers during the test on freeze-dried preparations are not regarded as visible particles.

V C Determination of Disintegration

Disintegration is provided to determine whether the oral solid preparations disintegrate or disperse into fragments or particles within a prescribed time when placed in a liquid medium under the prescribed experimental conditions.

Disintegration is considered to be achieved when no residue, except fragments of undissolved tablet coating or of capsule shell, remains on the screen of the test apparatus. Disintegration can be also considered to be achieved when the residue remained consists of a soft mass having no palpably, unmoistened core, or floats because of light weight.

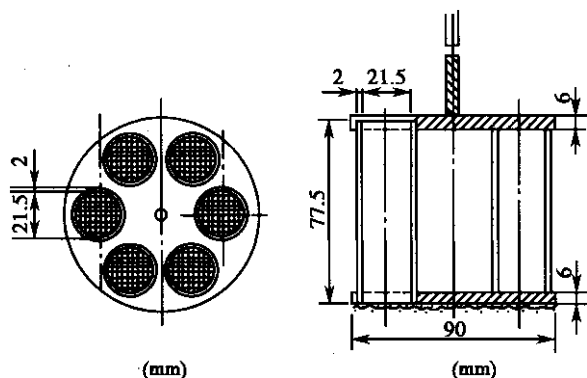
Disintegration is not required for the preparations which comply with the requirements for Dissolution, Drug release or Disintegration for Suppositories and Vaginal Tablets.

1. Tablets

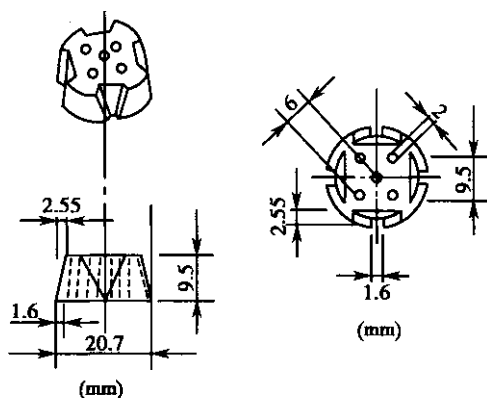
Apparatus The apparatus mainly consists of a basket-rack assembly with disks and a metallic device capable of raising and lowering the basket in the liquid medium at a constant rate between 30 and 32 cycles per minute through a distance of $55 \text{ mm} \pm 2 \text{ mm}$.

Basket-rack assembly The basket-rack assembly consists of six glass tubes, each $77.5 \text{ mm} \pm 2.5 \text{ mm}$ long, 21.5 mm in internal diameter with a wall of 2 mm in thickness. The tubes are held in a vertical position by two transparent plastic plates, each about 90 mm in diameter and 6 mm in thickness, with six holes, each about 26 mm in diameter. On the top of the upper plastic plate is a stainless-steel plate about 90 mm in diameter and 1 mm in thickness, with six holes each about 22 mm in diameter. Attached to the under surface of the lower plate is a disk of stainless-steel wire gauze about 90 mm in diameter, with apertures of 2.0 mm in internal diameter. A stainless-steel central shaft about

80 mm long is fixed with the upper plastic plate and stainless-steel plate. The stainless-steel plate, the two plastic plates and the wire gauze are fixed together by three screws.



Disks The disk is made of a smooth transparent plastic material having a specific gravity of 1.18-1.20 with $20.7 \text{ mm} \pm 0.15 \text{ mm}$ in diameter, and $9.5 \text{ mm} \pm 0.15 \text{ mm}$ in thickness. It has five holes with 2 mm in diameter, one of the holes is located in the center and the others at an equal distance of 6 mm from the central hole. Equally spaced on the sides of the disk are four V-shaped notches. The dimensions of each notch are such that the upper openings are 9.5 mm wide and 2.55 mm deep and the lower openings are 1.6 mm wide and 1.6 mm deep.



Procedure The basket is suspended in a water bath preferably using a 1000 ml of beaker, maintained at $37^\circ\text{C} \pm 1^\circ\text{C}$, the volume of the fluid in the vessel is adjusted appropriately so that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid and descends to a distance not less than 25 mm from the bottom of the vessel on the downward stroke.

Unless otherwise specified, place 1 tablet in each of the six tubes of the basket and operate the apparatus. All of the tablets shall disintegrate completely within 15 minutes. If 1 tablet fails to disintegrate completely, repeat the test on 6 additional tablets. All of the tablets shall comply with the requirements.

Film-coated tablets Carry out the test as described above. Replacing the water in the beaker with hydrochloric acid ($9 \rightarrow 1000$), all of the tablets shall disintegrate within 30 minutes. If any one of the tablets has not disintegrated, repeat the test on additional six tablets; all the tablets shall comply with the test.

Sugar-coated tablets Carry out the test as described above. All of the tablets shall disintegrate within 60 minutes. If any one of the tablets has not disintegrated, repeat the test on additional six tablets; all the tablets shall comply with the test.

Enteric-coated tablets Enteric-coated tablets are tested in the same way as described above using hydrochloric acid solution ($9 \rightarrow 1000$) as the immersion fluid. If the tablets show no evidence of cracking, disintegrating or softening in 2 hours. Remove the basket, wash the tablets with a small quantity of water, add a disk to each tube. Repeat the operation using phosphate BS (pH 6.8) as the immersion fluid, all tablets shall disintegrate completely within 1 hour. If 1 tablet fails to comply with the test, repeat the operation with additional 6 tablets. All the tablets shall comply with the test.

Buccal tablets Unless otherwise specified, buccal tablets are tested in the same way as described above. All of the tablets shall disintegrate or dissolve within 30 minutes. If any one of the tablets has not disintegrated, repeat the test on additional six tablets. All the tablets shall comply with the test.

Sublingual tablets Unless otherwise specified, sublingual tablets are tested in the same way as described above. All of the tablets shall disintegrate and dissolve within 5 minutes. If any one of the tablets has not disintegrated, repeat the test on additional six tablets. All the tablets shall comply with the test.

Soluble tablets Unless otherwise specified, soluble tablets are tested in the same way as described above maintained the temperature at $15-25^\circ\text{C}$. All of the tablets shall disintegrate and dissolve within 3 minutes. If any one of the tablets has not disintegrated, repeat the test on additional six tablets. All the tablets shall comply with the test.

Colon-located enteric-coated tablets Unless otherwise specified, colon-located enteric-coated tablets are tested in the same apparatus as described under the individual monograph. All of the tablets shall not release and disintegrate in the sulfuric acid solution ($9 \rightarrow 1000$) and the phosphoric acid buffer solution with pH value less than 6.8, but release and disintegrate in the phosphoric acid buffer solution with pH value between 7.8 and 8.0 within 1 hour, and the tablet core also disintegrates. If any one of the tablets has not disintegrated, repeat the test on additional six tablets. All the tablets shall comply with the test.

Effervescent tablets Effervescent tablets are tested in the following way. Place one tablet in a 250 ml beaker containing 200 ml of water maintained at 15-25°C, numerous gas bubbles are evolved. When the evolution of gas around the tablet or its fragments has ceased the tablets disintegrate, dissolve or disperse in the water so that no agglomerates of particles remain. Unless otherwise specified, the preparation being examined complies with the test if 6 tablets used in the test disintegrate in the manner prescribed within 5 minutes. If any one of the tablets has not disintegrated, repeat the test on additional six tablets. All the tablets shall comply with the test.

2. Capsules

Unless otherwise specified, hard capsules or soft capsules are tested as described above. If the capsules float on the surface of the water, a disk may be added. The hard capsules shall disintegrate within 30 minutes and the soft capsules disintegrate within 1 hour. If one of the capsules has not disintegrated, repeat the test on further six capsules, all the capsules shall comply with the test. For soft capsules, replacing the water in the beaker with simulated gastric fluid.

Unless otherwise specified, enteric capsules are tested in the same manner as described above using hydrochloric acid solution (9 → 1000) as the immersion fluid and the capsule shells show no evidence of cracking, disintegrating in 2 hours. Remove the basket, wash the capsules with a small quantity of water, add a disk to each tube. Repeat the operation using simulated intestinal fluid as the immersion fluid, all capsules shall disintegrate completely within 1 hour. If 1 capsule fails to comply with the test, repeat the operation with another 6 capsules. All the capsules shall comply with the test.

3. Dripping pills

Carry out the test with the same apparatus as described as tablets using the stainless steel wire gauze with apertures of 0.425 mm in internal diameter. Carry out the test as described above using 6 dripping pills, unless otherwise specified. All of the dripping pills disperse completely within 30 minutes, and the coated-dripping pills shall disperse completely within 60 minutes. If any one of the dripping pills has not dispersed completely, repeat the test on further six dripping pills, all the dripping pills shall comply with the test. Dripping pills made of gelatin matrix may be tested in simulated gastric fluid.

Annotation *Simulated gastric fluid* Add about 800 ml of water and 10 g of pepsin to 16.4 ml of dilute hydrochloric acid, mix well, dilute with water to produce 1000 ml.

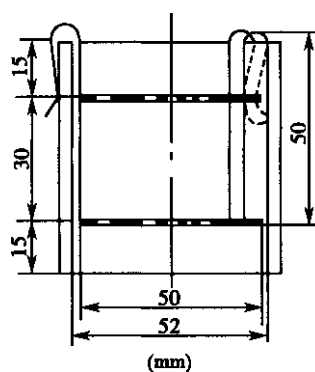
Simulated intestinal fluid See Phosphate-Pancreatin BS (pH 6.8) (Appendix XV D of Volume II).

V D Disintegration Test for Suppositories and Vaginal Tablets

The test for disintegration of suppositories and vaginal tablets determines whether a solid dosage form such as suppositories and vaginal tablets can disintegrate, soften, dissolve or disperse under the specified experimental conditions.

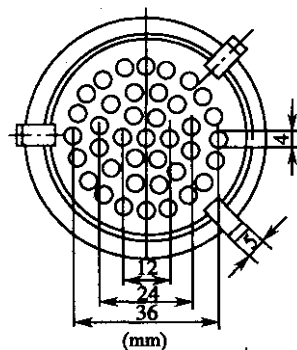
1. Suppositories

Apparatus The apparatus is composed of a transparent sleeve and a metal device.



Transparent sleeve The transparent sleeve is made of glass or suitable plastic, with a height of 60 mm, an internal diameter of 52 mm and an appropriate wall thickness.

Metal device The metal device consists of two stainless steel discs and three metal hooks. Each of the disc has a diameter of 50 mm and contains 39 holes, each 4 mm in diameter. The discs are separated by a distance of 30 mm. The metal device is attached to the outer sleeve by means of the three equally-spaced hooks.



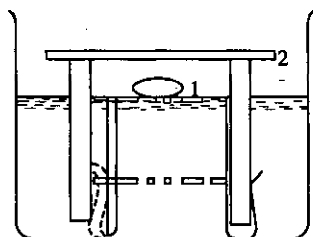
Procedure Take 3 suppositories being examined and allow to stand at room temperature for 1 hour. Place them on the lower discs of 3 metal devices respectively, then insert the devices into separate sleeves and fix them by means of the hooks. Unless otherwise specified, place each piece of the above

apparatus in 3 vessels separately, each containing not less than 4 litres of water at $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and fitted with a slow stirrer and a means of holding the apparatus vertically 90 mm below the surface of water, after each 10 minutes invert each apparatus without allowing it to emerge from the liquid.

Interpretation Unless otherwise specified, all of the fat-based suppositories shall disintegrate, soften or have no solid core offering resistance to pressure within 30 minutes; all of the water-soluble based suppositories shall completely dissolve within 60 minutes. If one of the suppositories fails to comply with the requirements, repeat the test on 3 additional suppositories and all of them shall comply with the requirements.

2. Vaginal tablets

Apparatus The apparatus is the same as that used for suppositories except that set the hook-end upside down in the vessel.



1—Vaginal tablet; 2—Glass plate;
3—Water surface

Procedure Adjust the water surface level until the holes of the upper metal disc are just covered by a uniform layer of water. Put 3 vaginal tablets being examined on separate upper discs and cover the apparatus with a glass plate to maintain appropriate humidity.

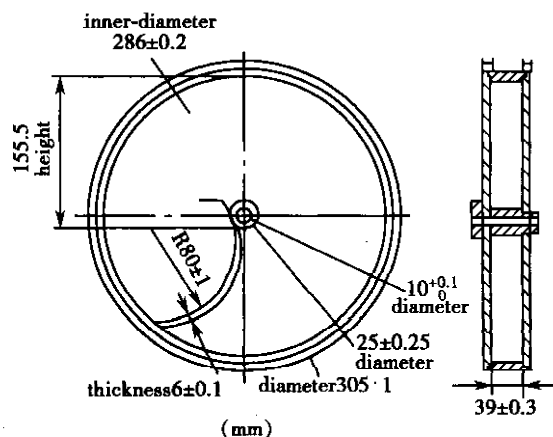
Interpretation Unless otherwise specified, all of the vaginal tablets shall dissolve or disintegrate into fragments and pass through the perforated plates or only remain a small amount of soft masses which have no solid core within 30 minutes. If one of the tablets fails to comply with the requirements, repeat the test on 3 additional tablets and all of them shall comply with the requirements.

V E Test for Tablet Friability

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets, and supplements other physical strength measurements, such as tablet crushing strength.

Apparatus Use a drum with an internal diameter

of about 286 mm and a depth of 39 mm made of a transparent synthetic polymer with polished internal surfaces, and not subject to static build-up. One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius of $80\text{ mm} \pm 1\text{ mm}$ that extends from the middle of the drum to the outer wall. The drum is attached to the horizontal axis of a device that rotates at $25 \pm 1\text{ r/min}$. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.



Tablet friability apparatus

Procedure For tablets weighing up to 0.65 g each, take a 6.5 g of sample; for tablets weighing over 0.65 g each, a 10-tablet sample is sufficient. Place the tablets on a No. 10 sieve and remove any loose dust with the aid of air pressure or a soft brush. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before and weigh.

Generally, the test is run once. If the results are doubtful or if the weight loss is greater than 1%, the test should be repeated twice and determine the mean of the three tests. A maximum weight loss of not more than 1% of the weight of the tablets being tested is considered acceptable and any tablets broken, chapped and smashed are not picked up.

If tablet size or shape causes irregular tumbling, adjust the drum so that its axis forms a 10° angle with the base and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

If tablet size or shape causes severe irregular tumbling in the drum, or tablets are produced by special procedure, this method are not suitable and may not be used to test the tablets.

In the cases of hygroscopic tablets, care must be taken to perform the test quickly enough to prevent moisture absorption (relative humidity is not more than 40%).

V F Test for Minimum Fill

The following tests and specifications apply to solid, semisolid, and liquid dosage forms packed in containers, except those preparations for which the tests for Weight Variation of Contents and Volume in Container are otherwise specified required or radio active pharmaceuticals.

Procedure

Gravimetric method (for containers labelled by weight)

Unless otherwise specified, select 5 containers (or 3 containers if the labelled quantity is more than 50 g), remove the cover and label. Thoroughly clean and dry the outside of the containers by suitable means, and weigh individually and accurately. Accurately remove the contents from each container, wash the container with a suitable solvent. Dry, and again weigh each empty container. Determine the

net weight of contents in each container and the average net weight of contents. The results comply with the requirements. If one container fails the requirements, a further 5 (or 3) containers may be tested individually and all must comply.

Volumetric method (for containers labelled by volume)

Unless otherwise specified, select 5 containers (or 3 containers if the labelled quantity is more than 50 ml). Open the containers with caution to avoid any loss of the contents. Take up individually the contents of each container into a dry, previously standardized syringe, or pour into a dry, previously standardized graduated cylinder, if the labelled quantity is more than 50 ml. For viscous liquids, keep the containers upside down for 15 minutes to pour the contents as completely as possible. Measure the volume of the contents in each container, calculate the average volume of contents. The results comply with the requirements. If one container fails the requirements, a further 5 (or 3) containers may be tested and all must comply.

Labelled quantity	Solids, semisolids, liquids		Viscous liquids (volumetric method)	
	Average quantity	Quantity in each container	Average quantity	Quantity in each container
Less than 20 g(ml)	Not less than the labelled quantity	Not less than 93% of the labelled quantity	Not less than 90% of the labelled quantity	Not less than 85% of the labelled quantity
20-50 g(ml)	Not less than the labelled quantity	Not less than 95% of the labelled quantity	Not less than 95% of the labelled quantity	Not less than 90% of the labelled quantity
More than 50 g(ml)	Not less than the labelled quantity	Not less than 97% of the labelled quantity	Not less than 95% of the labelled quantity	Not less than 93% of the labelled quantity

V G Determination of Particle Size

The following procedures are used for the determination of particle size or particle distribution. The method 1 and method 2 are used for the determination of particle size or size limit in pharmaceutical preparation.

Method 1 (microscopy)

The particle size determined by this method is expressed as the length of particles observed under a microscope.

Standardization of ocular micrometer Standardization of ocular micrometer is carried out to ascertain the value of each unit of the ocular micrometer for each optical combination (objective, ocular, and tube length).

Place the stage micrometer on the stage of the microscope. Focus the illuminator and the scale of the stage micrometer. Adjust stage micrometer until the image is in the centre of the field of view. Take out the ocular. Unscrew the cover of the

ocular, and insert the ocular micrometer within the tube of the ocular by placing it on the shelf which is about halfway between the upper and lower lenses of the ocular. The engraved surface must be faced upper. Screw the cover of the ocular, and place the ocular back to the tube of the ocular. The image of the stage micrometer and the scale of the ocular micrometer can be observed in the same field of view. Make the lines on the two micrometers parallel each other and the extreme left line (marking 0) on each scale coincide by adjusting the stage micrometer and rotating the ocular. Find the second lines which coincide, and count the number of included units of each micrometer and calculate the ratio. The value (μm) of each unit of the ocular micrometer for the objective, with which it is used, will be judged based on the ratio. As the value for each unit of the stage micrometer is 10 μm , the value of each unit of the ocular micrometer can be calculated by the following equation:

$$\frac{10 \times \text{the number of included units of the stage micrometer}}{\text{the number of included units of the ocular micrometer}}$$

When two objectives with different magnification are to be used, it is necessary to ascertain the value of each unit of the ocular micrometer for each

objective with which it is used.

Procedure Thoroughly mix the substance being examined. For the substance with high viscosity, a suitable quantity of glycerin solution (1→2) specified in the individual monograph may be added. Introduce 1-2 drops of the substance being examined onto a slide as specified in the appendix of the dosage form or in the individual monograph, cover a cover glass, and press gently to make the particles spread uniformly. Prevent formation of bubbles. For the substance being examined which is semisolid, it can be spread directly onto a slide. Immediately examine the slide under a microscope, in the whole field of view, using 50-100× magnification. No agglomeration occurs and none of the particles has a dimension of 50 μm or greater than it specified in the individual monograph. Examine the slide again under a microscope, in the field of view specified in the appendix of the dosage form or in the individual monograph, using 200-500× magnification. Record the total number of particles observed and the number of particles having the specified dimension. Calculate the percentage.

Method 2 (sieving)

Single sieve Place a quantity of the substance being examined, as specified in the individual monograph, upon the specified No. sieve with a close-fitting receiving pan and cover. Shake the sieve in a rotary horizontal direction for not less than 3 minutes, and gently tap on the sieve frequently in vertical direction. Weigh accurately the amount in the receiving pan, and calculate the percentage.

Two sieves Place the accurately weighed content of 5 single-dose units or of 1 multi-dose unit upon the sieve specified in the appendix of the dosage form or in the individual monograph. Shake the sieve in a left-to-right horizontal direction by gently tapping on the sieve for 3 minutes. Weigh accurately the amount remaining on the smaller No. sieve and passing through the larger No. sieve. Calculate the percentage of the fraction.

V H Determination of Osmolality

The phenomenon that a solvent diffuses through a semi-permeable membrane from a solution of low concentration to a higher concentration solution is called osmosis. The pressure that is needed to prevent osmosis is called osmotic pressure. Biomembrane, such as cell membrane or capillary wall of the body, has the properties of a semi-permeable membrane, thus the osmotic pressure must be considered for injection and eye drops. The declaration of osmolar concentration on the

label of intravenous replenishment liquid, nutrient (s), electrolyte (s) or osmotic diuretic agents, such as Monnitol Injection, are required to inform the clinical doctors.

The units of osmolar concentration are usually expressed as milliosmoles (mOs mol) of solute per kilogram of solution. The ideal milliosmole concentration may be determined according to the formula:

$$\text{Milliosmole concentration (mOs mol/kg)} = \frac{\text{weight of solute (g/kg)}}{\text{molecular weight (g)}} \times n \times 1000$$

Where n is the number of ions or chemical species produced when the solute is dissolved. In ideal solutions, for example, $n=1$ for glucose, $n=2$ for sodium chloride or magnesium sulfate, $n=3$ for calcium chloride, $n=4$ for sodium citrate. Deviation from ideal condition is usually slight in solutions within the physiological range and for more dilute solutions, but for highly concentrated solutions, the actual osmolarities may be appreciably lower than ideal values. For example, the ideal osmolality of 0.9% Sodium Chloride Injection is $2 \times 1000 \times 9/58.4 = 308$ mOs mol/kg, but in fact, its n is slightly less than 2, and the actual measured osmolality is 286 mOs mol/kg. The theoretical osmolality of a complex mixture, such as Protein Hydrolysate Injection, can not be readily calculated, so the actual measured value is usually used.

Apparatus It is difficult to directly measure osmolality, but more convenient to measure the freezing point, so osmolality is often determined by measuring the depression of freezing point. In ideally dilute solution, the depression of freezing point shall conform to the following equation: $\Delta T_f = K_f \cdot m$, where ΔT_f is the depression value of freezing point, K_f is the depression constant of freezing point ($K = 1.86$ when the solvent is water), m is the weight mole concentration. Osmolality is calculated by $P_o = K_o \cdot m$, where P_o is osmolality, K_o is the constant of osmolality, and m is the weight mole concentration. Due to the same meaning of concentration in the two formula mentioned above, osmolality can be determined by measuring the depression of freezing point. Osmometer is generally designed according to the principle of the depression of freezing point. An osmometer consists of a test tube containing the solution to be examined, a cooling system with temperature regulator and a pair of resistor sensitive to temperature (thermistors). When measuring, immerse the sensor into the solution in the glass tube, lower the tube into the cooling system and the temperature of the cooling system is decreased until the solution is super-cooled. The measured freezing point is converted to a measurement value by instrument.

Procedure Determine the zero point by using a certain volume of water (prepared freshly), calibrate the osmometer using reference solutions whose osmolality range is close to that expected for the solution being examined, then measure the osmolality of the solution being examined. When the osmolality of the solution being examined is beyond the range limit of the osmometer, dilute it with appropriate solvent to a measurable range of osmolality. If the sample is a solid, dissolve it in an appropriate solvent and then determine.

Preparation of reference solution for calibration of osmometer

Dissolve a quantity, accurately weighed, of sodium chloride (primary reference substance) previously dried for 40 to 50 minutes at 500-650°C and cooled to room temperature in a desiccator contain in silica gel, as described in the Table, as required, in 1 kg of water, mix well.

Reference solution for calibration of osmometer

Weights of sodium chloride in 1 kg of water(g)	Milliosmole concentration (mOsmol/kg)	The depression of freezing point (°C)
3.087	100	0.186
6.260	200	0.372
9.463	300	0.558
12.684	400	0.744
15.916	500	0.930
19.147	600	1.116
22.380	700	1.302

Determination of milliosmole concentration ratio

The ratio of milliosmole concentration of the test sample to that of 0.9% (W/V) sodium chloride solution is called milliosmole concentration ratio. Determine the milliosmole concentration of the test sample (O_T) and that of the reference solution (O_S) respectively, calculate the milliosmole concentration ratio by the following equation:

$$\text{milliosmole concentration ratio} = \frac{O_T}{O_S}$$

Preparation of reference solution for measurement of milliosmole concentration ratio

Weigh accurately 0.900 g of sodium chloride (primary reference substance), previously dried for 40-50 minutes at 500-650°C and cooled to room temperature in a desiccator containing silica gel, into a 100 ml volumetric flask, dissolve in water and dilute to volume, mix well.

For the preparation of injections or eye drops containing sodium chloride, osmolality is often measured instead of the assay of sodium chloride if the function of sodium chloride is mainly to regulate the osmotic pressure.

Appendix VI

VI A Determination of Nitrogen (Semi-micro Method)

The method is based on the principle that nitrogen-containing organic substances form ammonium sulfate after digestion with sulfuric acid. Ammonium sulfate is decomposed by sodium hydroxide and ammonia is released. The released ammonia is evaporated by steam distillation and absorbed by boric acid solution to form ammonium borate. After titration with strong acid, the nitrogen content of test sample can be calculated from the volume of strong acid consumed.

Reagent

(1) Digestion mixture

Prepare by grinding and mixing 10 g of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and 100 g of potassium sulfate thoroughly in an agate mortar.

(2) Mixed indicator solution

Prepare by mixing five volumes of 0.2% bromocresol green ethanol solution and two volumes of 0.1% methyl red ethanol solution thoroughly.

(3) 2% boric acid absorption solution

Dissolve 20 g of boric acid in water and dilute to 1000 ml. Add 10 ml of mixed indicator solution and mix well.

(4) Sulfuric acid VS (0.05 mol/L)

Add slowly 3 ml of sulfuric acid to a quantity of water. Cool the mixture to room temperature and dilute to 1000 ml with water.

Weigh accurately 0.15 g of dehydrated sodium carbonate primary standard, which has been dried at 270-300°C to constant weight, and dissolve in 50 ml of water. Add ten drops of mixed indicator solution. Titrate with the above sulfuric acid solution until the colour of the solution changes from green to purple. Boil for 2 minutes and cool down to room temperature. Continue the titration until the colour of the solution changes from green to dark purple. One ml of 0.05 mol/L sulfuric acid VS is equivalent to 5.30 mg of dehydrated sodium carbonate. The concentration of the sulfuric acid solution is calculated according to the volume of the sulfuric acid solution consumed and the weight of dehydrated sodium carbonate taken.

(5) Sulfuric acid VS (0.005 mol/L)

Transfer accurately 100 ml of 0.05 mol/L sulfuric

acid VS to a 1000 ml volumetric flask. Dilute to volume with water and mix thoroughly.

Procedure

Measure accurately a quantity of test sample (containing about 1.0-2.0 mg of nitrogen) and put into a Kjeldahl flask. Add approximately 0.3 g of digestion mixture and 1 ml of sulfuric acid. Digest the mixture by heating to form a clear bluish-green colour solution and continue the digestion for 60 minutes.

Measure 10 ml of 2% boric acid absorption solution and put into a 100 ml conical flask. Immerse the condenser tip of Kjeldahl distillation apparatus into 2% boric acid absorption solution. Then, pour the digested test sample into the Kjeldahl distillator. Wash the Kjeldahl flask 3-4 times with water. All the washings are transferred to the distillator. Add 5 ml of 50% sodium hydroxide solution and distill until the total volume of the received liquid reaches 35-50 ml. Lower the receiving bottle to let the condenser tip rises above the surface of liquid. Continue the distillation for 1 minute and wash the tip of condenser with a small volume of water. The received liquid is then titrated with 0.005 mol/L sulfuric acid VS until the colour of the liquid changes from green to pale purple. The titration result is calibrated with a blank test.

Calculate the result according to the following equation:

$$\text{Total nitrogen content of test sample (mg/ml)} = (V_x - V_o) \times C \times 14.01 \times n \times 2 / V$$

Where: V_x = Titer of sample, ml;

V_o = Titer of blank, ml;

C = Concentration of sulfuric acid VS, mol/L;

n = Dilution factor of sample;

V = Volume of sample, ml.

The value of 14.01 is the relative atomic weight of nitrogen.

[Notes]

(1) The distillator shall be washed by distillation for more than 15 minutes before distillation.

(2) Several drops of sulfuric acid and methyl red indicator solution shall be added to the steam generator until the colour of water is distinctly red.

(3) The test may also be carried out with an automatic Kjeldahl nitrogen determination apparatus.

VI B Determination of Protein Content

Method 1 Kjeldahl Method

The protein content in test sample is calculated through the determination of total nitrogen content and the determination of non-protein nitrogen content after the protein is removed by tungstic acid precipitation.

Preparation of test sample solution

(1) Preparation of solution for total nitrogen determination

Dilute volumetrically 1 ml of test sample with physiological saline to a nitrogen content of about 1 mg per ml.

(2) Preparation of solution for non-protein nitrogen determination

Measure accurately 2 ml of test sample and mix with 14 ml of water. Add 2 ml of 10% sodium tungstate solution and 2 ml of sulfuric acid solution (1.86→100). Mix thoroughly. Allow the mixture to stand for 30 minutes and filter. Preserve the filtrate for determination.

Procedure

Measure accurately 1 ml of solution for total nitrogen determination and put into a Kjeldahl flask. Determine the total nitrogen content of the test sample according to nitrogen determination method (Appendix VI A). A blank control test is carried out at the same time.

Measure accurately 5 ml of solution for non-protein nitrogen determination and put into a Kjeldahl flask. Determine the non-protein nitrogen content of the test sample according to nitrogen determination method (Appendix VI A).

Result calculation

$$C_{TN} \text{ (mg/ml)} = (V_{X1} - V_0) \times C \times 14.01 \times n \times 2 / V_1$$

$$C_{NPN} \text{ (mg/ml)} = (V_{X2} - V_0) \times C \times 14.01 \times n \times 2 / V_2$$

$$C_{PN} \text{ (mg/ml)} = C_{TN} - C_{NPN}$$

$$\begin{aligned} \text{Protein content of test sample, \% (g/ml)} \\ = C_{PN} \times 6.25 \times 100 / 1000 \end{aligned}$$

Where: C_{TN} = Total nitrogen content of test sample, mg/ml;

C_{NPN} = Non-protein nitrogen content of test sample, mg/ml;

V_{X1} = Titer of test sample solution for total nitrogen determination, ml;

V_{X2} = Titer of test sample solution for non-protein nitrogen determination, ml;

V_0 = Titer of blank, ml;

C = Concentration of sulfuric acid VS, mol/L;

C_{PN} = Protein nitrogen content of test sample, mg/ml;

n = Dilution factor of test sample;

V_1 = Volume of test sample solution for total nitrogen determination, ml;

V_2 = Volume of test sample solution for non-protein nitrogen determination, ml.

The value of 14.01 is the relative atomic weight of nitrogen;

The value of 6.25 is a constant (1 g of nitrogen is equivalent to 6.25 g protein).

[Note]

(1) If the protein content of the test sample is more than 10% (g/ml), the dilution factor of test sample upon deproteinization shall increase properly. Meanwhile, the amounts of 10% sodium tungstate solution and sulfuric acid solution shall increase proportionally so as to maintain the concentration of tungstic acid at 1% in the solution.

(2) Same blank control may be used for total nitrogen and non-protein nitrogen determinations.

Method 2 Lowry Method

The method is used for the micro-determination of protein content. Protein can form copper-protein complex in alkaline solution and the complex reacts with phenol reagent to form a blue compound. The absorbance of the compound at 650 nm is directly proportional to protein content. The protein content of test sample is calculated according to the absorbance.

Reagent

(1) Phenol reagent

Weigh 100 g of sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) and 25 g of sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), and put into a 1500 ml distillation flask. Add 700 ml of water, 50 ml of 85% phosphoric acid and 100 ml of hydrochloric acid. Fix a reflux condenser on top of the flask by using a cork or rubber stopper (wrapped with tinfoil) and reflux for 10 hours by gently boiling. Disconnect the reflux condenser. Add 150 g of lithium sulfate, 50 ml of water and several drops of bromine water. Boil about 15 minutes to expel excessive bromine. Cool down. Dilute to 1000 ml with water and filter. A phenol reagent stock solution is prepared.

The acidity of phenol reagent stock solution is titrated with 0.5 mol/L sodium hydroxide VS. The phenol reagent stock solution is then diluted with water to a concentration equivalent to 1 mol/L hydrochloric acid. This solution is phenol reagent.

(2) Alkaline copper solution

Mix 0.5 ml of 0.1 mol/L potassium tartrate solution, 0.5 ml of 0.04 mol/L cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution, 25 ml of 4% sodium carbonate solution and 25 ml of 0.8% sodium hydroxide solution thoroughly. The solution shall be prepared just before use.

(3) Preparation and calibration of 0.5 mol/L sodium hydroxide VS

Dissolve a quantity of sodium hydroxide in water to make a saturated solution. Store in a polyethylene bottle after cooling down. Allow to stand for several days until the supernatant is clear. Dilute

28 ml of the clear saturated sodium hydroxide solution with freshly boiled and cooled water to 1000 ml. Mix well.

Weigh accurately 3 g of potassium biphthalate primary standard, which has been dried to constant weight at 105°C. Add 50 ml of freshly boiled and cooled water. Shake well to make the solid dissolved as much as possible. Add two drops of phenolphthalein indicator solution. Titrate with the sodium hydroxide solution until the end point is near. The potassium biphthalate shall be completely dissolved. Continue the titration procedure until a distinct pink colour appears. One milliliter of the sodium hydroxide VS is equivalent to 102.1 mg of potassium biphthalate. The concentration of sodium hydroxide solution is calculated according to the weight of potassium biphthalate taken and the volume of volumetric solution consumed.

Preparation of protein standard solution

A protein standard stock solution is prepared by accurately diluting one container of human albumin standard with water to 1 mg of protein per ml. A protein standard solution of 100 µg of protein per ml is prepared by transferring accurately 2.5 ml of protein standard stock solution to a 25 ml volumetric flask and diluting to volume with water. Mix well.

Procedure

Measure accurately a volume of test sample (containing about 50 µg of protein) and put into a test tube. Make up to 1 ml with water. Add 5 ml of alkaline copper solution and mix well. Allow the mixture to stand for 10 minutes at room temperature. Add quickly 0.5 ml of phenol reagent and mix well. Allow the mixture to stand for 30 minutes at room temperature. Read the absorbance at 650 nm according to ultraviolet-visible spectrophotometry (Appendix II A). If the mixture is turbid after colour development, centrifuge at 3000 r/min for 15 minutes and read the absorbance of supernatant.

Measure accurately 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of protein standard solution and put into a series of test tubes separately. For each tube, make up the volume to 1 ml with water and then proceed with the same procedure as that for test sample starting from "add 5 ml of alkaline copper solution". A standard curve is obtained. Carry out a blank control test by measuring accurately 1 ml of water in a test tube and proceed with the same procedure as that for test sample starting from adding 5 ml of alkaline copper solution.

A regression equation is obtained by regressing the concentrations of protein standard with the corresponding absorbance. The protein content of test sample is obtained by inserting its absorbance into the regression equation.

Method 3 Biuret Method

The method is based on the principle that peptide bonds of protein react with Cu^{++} and form a purple complex in alkaline solution, and the colour intensity of the complex is directly proportional to the protein content. The protein content of test sample is determined against a protein standard solution by using ultraviolet-visible spectrophotometry (Appendix II A).

Reagent

Biuret reagent

Dissolve 3.0 g of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 9.0 g of potassium sodium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$), 5.0 g of potassium iodide and 24 g of sodium hydroxide in water and dilute to 1000 ml. Mix well.

Preparation of protein standard solution

Measure accurately a quantity of human albumin standard and dilute volumetrically with water to a concentration of 50 mg of protein per ml.

Preparation of test sample solution

Dilute volumetrically a quantity of test sample with water to about 50 mg of protein per ml.

Procedure

Measure accurately 0.05 ml of test sample solution and 0.05 ml of protein standard solution, and put into two test tubes separately. To each test tube, add 4.0 ml of biuret reagent and mix well. Allow the mixture to stand in 37°C water bath for 30 minutes. Read the absorbance at 540 nm by ultraviolet-visible spectrophotometry (Appendix II A). Carry out a blank control test by measuring accurately 0.05 ml of water to a test tube and proceed with the same procedure starting from "add 4.0 ml of biuret reagent".

Calculate the result according to the following equation:

$$\text{Protein content (mg/ml)} = (A_1/A_2) \times C \times n$$

Where: A_1 = Absorbance of sample solution;

A_2 = Absorbance of protein standard solution;

C = Concentration of protein standard solution, mg/ml;

n = Dilution factor of test sample.

[Note]

The determination range of the method is 1-10 mg of protein.

VI C Determination of Sialic Acid Content (Resorcinol Colorimetry)

The method is based on the principle that conjugated sialic acid becomes free acid after acid hydrolysis. Free sialic acid reacts with resorcinol to form a coloured compound. The sialic acid

content is determined after the coloured compound is extracted with organic acid.

Preparation of 200 µg/ml sialic acid reference solution

Weigh accurately 10.52 g of sialic acid CRS (1 µg is equivalent to 3.24 nmol) and dissolve in a small amount of water. Transfer totally to a 10 ml volumetric flask and dilute to volume with water. Mix well and a 1 mg/ml sialic acid reference stock solution is prepared. Dispense the stock solution to the volume for one test and store at -70°C. The validity period of the dispensed standard is 12 months. It can only be frozen and thawed once. The validity period is 2 weeks if it is stored at 4°C. Measure accurately 1 ml of sialic acid reference stock solution (1 mg/ml) and put into a 5 ml volumetric flask. Dilute to volume with water. The sialic acid reference solution at a concentration of 200 µg per ml shall be prepared just before use.

Procedure

Dilute a quantity of test sample with water to a protein concentration of about 0.2-0.4 mg per ml. To a set of glass test tubes, add sialic acid reference solution, test sample solution and water respectively according to the volume listed in the following table, mix well. To each tube, add 1 ml of resorcinol-hydrochloric acid solution (Mix 2.5 ml of 2% resorcinol solution and 62.5 µl of 0.1 mol/L cupric sulfate solution with 20 ml of 25% hydrochloric acid. Dilute to 25 ml with water and mix well. This solution is prepared within 4 hours prior to the test). Stopper the tubes and heat in a boiling water bath for 30 minutes with their liquid surface level about 2 cm lower than that of water bath. Take the test tubes out of boiling water bath and cool in an ice bath for 3 minutes with shaking. Then, add 2 ml of butyl acetate-butanol solution (Mix four volumes of butyl acetate with one volume of butanol. Store at room temperature and use within 12 hours) to each tube and mix well. Allow the mixture to stand at room temperature for 10 minutes. Read the absorbance at 580 nm by ultraviolet-visible spectrophotometry (Appendix II A).

Tube	Sialic acid CRS tubes						Sample tube
	Blank	2 µg	4 µg	5 µg	6 µg	8 µg	
Sialic acid reference solution(µl)		10	20	25	30	40	
Water(µl)	100	90	80	75	70	60	
Test sample solution(µl)							100

A linear regression equation is obtained by regressing the concentrations of sialic acid reference solution with the corresponding absorbance (the correlation coefficient shall be not less than 0.99). The absorbance of sialic acid (5 µg)

is obtained from the linear regressing equation. The sialic acid content of test sample is calculated by the following equation.

$$\text{Sialic acid content of test sample (mol/mol Protein)} = A_2 \times 5 \times 3.24 \times W \times D / (A_1 \times P \times 100)$$

Where: A_1 = Absorbance of sialic acid CRS (5 µg);

A_2 = Absorbance of test sample;

D = Dilution factor of test sample;

P = Protein content of test sample, µg/µl;

W = Amount of 1 nmol erythropoietin is equivalent to 18.2 µg (sugar component is not included).

VI D Determination of Residual Ethanol Content (Conway Diffusion Method)

The method is based on the principle that ethanol is evaporated from saturated sodium carbonate solution upon heating. After the evaporation ethanol is absorbed by potassium dichromate solution, and a yellow-green to green colour appears. The residual ethanol content in blood products is determined by spectrophotometry.

Procedure

Spread a thin layer of vaseline evenly on the outside rim of a Conway Disk. Measure accurately 2.0 ml of potassium dichromate-sulfuric acid solution (Dissolve 3.7 g of potassium dichromate in 150 ml of water. Add 280 ml of concentrated sulfuric acid slowly. Cool down and dilute to 500 ml with water. Mix well) and put into the inner circle of the disk. Measure accurately 1.5 ml of test sample solution and 1.5 ml of saturated sodium carbonate solution [Measure a quantity of sodium carbonate ($\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$) and mix with the same quantity of water. Mix thoroughly and preserve the supernatant for use.] and add into the outer circle of the disk. Put on a glass immediately with its rough side downward to seal the disk tightly. Mix well by shaking carefully. React at 80°C for 30 minutes. Determine the absorbance (A_1) of the solution in the inner circle at 650 nm by ultraviolet-visible spectrophotometry (Appendix II A).

An ethanol reference solution is prepared by measuring accurately a quantity of absolute ethanol and dilute with water to a concentration of 0.25 mg per ml. Measure accurately 1.5 ml of the ethanol reference solution and carry out the same procedure as that for test sample solution. The absorbance obtained is A_2 . The reading of A_1 shall be not greater than A_2 .

VI E Determination of Free Histamine Phosphate in Human Histamine Immunoglobulin

The method is based on the principle that histamine phosphate reacts with o-phthaldialdehyde in alkaline solution to form a fluorescence derivative. The free histamine phosphate content in human histamine immunoglobulin is determined with fluorometry.

Preparation of histamine phosphate reference solution

Weigh accurately 7 mg of histamine phosphate CRS and dissolve in a small volume of 0.1 mol/L hydrochloric acid. Transfer totally to a 25 ml volumetric flask and dilute to volume with 0.1 mol/L hydrochloric acid. Mix well and a stock histamine phosphate reference solution is prepared. Store at -20°C for use. On the date of test, measure accurately 0.1 ml of stock histamine phosphate reference solution and put into a 100 ml volumetric flask. Dilute to volume with 0.1 mol/L hydrochloric acid. A histamine phosphate reference solution is prepared.

Preparation of test sample solution

Mix 0.5 ml of test sample with 1.2 ml of water. Add 0.3 ml of 25% trichloroacetic acid and mix well. Centrifuge at 4000 r/min for 10 minutes. The supernatant is taken as test sample solution.

Procedure

Measure 1.6 ml of test sample solution and put into a test tube. Add 1.5 g of sodium chloride, 4.0 ml of n-butanol and 0.2 ml of 2.5 mol/L sodium hydroxide solution. Mix instantly for 5 minutes. Allow the mixture to stand for the separation of liquids. Measure 3.6 ml of the nbutanol phase and put into a test tube containing 1.2 ml of 0.1 mol/L hydrochloric acid solution and 2.0 ml of n-heptane. Oscillate for 5 minutes and discard the organic phase. Mix 1.0 ml of the aqueous phase with an equal volume of water. Add 0.5 ml of 0.4 mol/L sodium hydroxide solution. Mix well and add quickly 0.1 ml of 0.1% o-phthaldialdehyde-methane solution. Mix well instantly. Allow the mixture to stand at $21-22^{\circ}\text{C}$ for 10 minutes. Stop the reaction by adding 0.5 ml of 0.5 mol/L hydrochloric acid solution. Transfer 200 μl of the reaction mixture to a well of microtiter plate. Measure the intensity of fluorescence light at an excitation wavelength of 350 nm and an emission wavelength of 450 nm with a fluorophotometer.

Measure accurately 1.0 ml, 0.8 ml, 0.6 ml, 0.4 ml, 0.2 ml, 0.1 ml, 0.05 ml and 0.025 ml of histamine phosphate reference solution and put into a series of test tubes separately. Make up the volume of each tube to 1.0 ml with 0.1 mol/L

hydrochloric acid. To each tube, add 0.5 ml of water and 0.1 ml of 25% trichloroacetic acid solution. Mix well and add 1.5 g of sodium chloride to each tube. Carry out the same procedure as that for test sample solution starting from "add... 4.0 ml of n-butanol".

A regression equation is obtained by regressing the concentrations of histamine phosphate reference solution with the corresponding fluorescence intensity. The alkaline base concentration of test sample solution (G, ng/ml) is obtained by inserting its fluorescence intensity into the regression equation. The free histamine content of test sample is calculated by the following equation.

Free histamine content of test sample (ng/ml) = $G \times 2.76 \times 2.5$

The value of 2.5 is the dilution factor of test sample.

[Note]

The relative molecular weight of histamine phosphate is 307.148. The concentration of reference solution is calculated as alkaline base and the relative molecular weight ratio of alkaline base to histamine phosphate is 1 : 2.76.

VI F Determination of O-Acetyl Content

Reagent

(1) 2 mol/L hydroxylamine hydrochloride solution
Dissolve 13.9 g of hydroxylamine hydrochloride in water and dilute to 100 ml. Store in a cold place.

(2) 3.5 mol/L sodium hydroxide solution
Dissolve 14.0 g of sodium hydroxide in water and dilute to 100 ml.

(3) 4 mol/L hydrochloric acid solution
Dilute 33.3 ml of hydrochloric acid to 100 ml with water.

(4) 0.37 mol/L ferric chloride-hydrochloric acid solution
Dissolve 10.0 g of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in 0.1 mol/L hydrochloric acid solution and dilute to 100 ml.

(5) Basic hydroxylamine solution
Mix 2 mol/L hydroxylamine hydrochloride solution with an equal volume of 3.5 mol/L sodium hydroxide solution. Use within 3 hours.

Preparation of reference solution

Weigh accurately 22.7 mg of acetylcholine chloride (or 28.3 mg of acetylcholine bromide), which has been dried to constant weight, and dissolve in a small volume of 0.001 mol/L sodium acetate solution (pH 4.5). Transfer totally to a 50 ml volumetric flask and dilute to volume with 0.001 mol/L sodium acetate solution. Mix well.

Preparation of test sample solution

Dilute the test sample with water to an O-acetyl

concentration of 0.5-2.5 mmol/L.

Procedure

Measure accurately 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of 2.5 mmol/L acetylcholine chloride (or acetylcholine bromide) reference solution and put into a series of test tubes separately. Make up the volume of each tube to 1.0 ml with water and add 2 ml of freshly prepared basic hydroxylamine solution. Mix well, allow to stand for 4 minutes at room temperature. To each tube, add 1 ml of 4 mol/L hydrochloric acid solution to adjust the pH to 1.2 ± 0.2 and mix well. Then, add 1 ml of 0.37 mol/L ferric chloride-hydrochloric acid solution and mix well again. Read the absorbance at 540 nm by ultraviolet-visible spectrophotometry (Appendix II A). Corresponding blank controls are prepared by measuring accurately another set of 2.5 mmol/L acetylcholine chloride (or acetylcholine bromide) reference solution and putting into another series of test tubes separately. Carry out the blank control tests with the same procedures starting from "make up the volume of each tube to 1.0 ml with water" except that the order of adding basic hydroxylamine hydrochloride solution and 4 mol/L hydrochloric acid solution is reversed. Transfer accurately 1 ml of test sample solution to a test tube. Carry out the same procedure as that for CRS starting from "add 2 ml of freshly prepared basic hydroxylamine solution". Carry out a blank control test for test sample by transferring accurately 1 ml of test sample solution to another test tube. Other procedures are the same as that for test sample solution except that the order of adding basic hydroxylamine hydrochloride solution and 4 mol/L hydrochloric acid solution is reversed. Subtract the absorbance of corresponding blanks from that of CRS tubes respectively. A linear regression equation is obtained by regressing the volume of reference solutions with the corresponding absorbance after blank correction. Subtract the absorbance of test sample blank from that of the test sample tube and insert into the regression equation. Calculate the volume of test sample equivalent to the volume of reference solution (E , ml).

O-acetyl content of test sample solution (mmol/L) = $E \times 2.5$.

The value of 2.5 is the acetylcholine concentration of reference solution in mmol/L.

VI G Determination of Residual Polyethylene Glycol Content

The method is based on the principle that polyethylene glycol reacts with barium and iodine ions and forms a complex. The polyethylene glycol content is determined by spectrophotometry.

metry.

Procedure

A test sample solution is prepared by diluting the test sample with water to a protein concentration of not more than 1%. Measure accurately 1.0 ml of the test sample solution and put into a test tube. Add 5.0 ml of 0.5 mol/L perchloric acid and mix well. Allow the mixture to stand at room temperature for 15 minutes and centrifuge at 4000 r/min for 10 minutes. Mix 4.0 ml of the supernatant with 1.0 ml of 5% barium chloride solution (Dissolve 5 g of powdered barium chloride in water and dilute to 100 ml) and 0.5 ml of 0.1 mol/L iodine solution (Dissolve 2.0 g of potassium iodide with a small amount of water. Then, add 1.3 g of iodine and dilute to 50 ml with water.) Mix well and react at room temperatures for 15 minutes. Read the absorbance at 535 nm by ultraviolet-visible spectrophotometry (Appendix II A). A blank control test is carried out with the same procedure using 1 ml of water instead of test sample solution.

Weigh accurately 0.100 g of polyethylene glycol (relative molecular weight 4000 or 6000) and dissolve in water to prepare a polyethylene glycol reference stock solution at a concentration of 100 μg per ml.

Prepare the reference solutions at the concentration of 10-50 $\mu\text{g}/\text{ml}$ according to the following table.

Polyethylene glycol($\mu\text{g}/\text{ml}$)	10	20	30	40	50
Polyethylene glycol reference stock solution(ml)	0.2	0.4	0.6	0.8	1.0
1% Protein solution(ml)	0.2	0.2	0.2	0.2	0.2
Water(ml)	1.6	1.4	1.2	1.0	0.8

Transfer 1.0 ml of each of the polyethylene glycol reference solution to a series of test tubes separately. To each tube, add 5.0 ml of 0.5 mol/L perchloric acid and mix well. Carry out the same procedure as that for test sample solution starting from "allowing the mixture to stand at room temperature for 15 minutes".

Result calculation

A linear regression equation is obtained by regressing the concentration of polyethylene glycol reference solutions with the corresponding absorbance. The polyethylene glycol concentration F ($\mu\text{g}/\text{ml}$) of the test sample solution is obtained by inserting the absorbance of the test sample solution to the linear regression equation.

Polyethylene glycol concentration of test sample (g/L) = $F \times G \times 10^3$.

Where; F = Polyethylene glycol content of test sample solution, $\mu\text{g}/\text{ml}$;
 G = Dilution factor of test sample.

[Note]

(1) The whole course of absorbance reading shall

be completed within 15-45 minutes after the addition of reagents, otherwise the result will be influenced.

(2) The sensitivity of this method increases along with the increase of relative molecular weight of polyethylene glycol.

(3) The protein solution used for the preparation of 1% protein solution shall not contain polyethylene glycol.

VI H Determination of Residual Polysorbate 80 Content

The method is based on the principle that polyethoxylated radicals in polysorbate 80 react with cobaltic ammonium thiocyanate to form a blue complex, which is soluble in dichloromethane. The polysorbate 80 content is determined by spectrophotometry.

Procedure

Measure accurately 1.0 ml of test sample and put into a centrifugal tube. Add 5 ml of saturated sodium chloride ethanol solution and mix well. Centrifuge at 3000 r/min for 10 minutes and collect the supernatant. Wash the wall of tube carefully with 1.0 ml of saturated sodium chloride ethanol solution, and pool the washing and supernatant. Centrifuge at 3000 r/min for 10 minutes again. Allow the supernatant to stand in 55°C water bath and concentrate to a volume of about 0.1-0.5 ml with blowing air. Dissolve with 1 ml of water. Add accurately 2.0 ml of dichloromethane and 3.0 ml of cobaltic ammonium thiocyanate solution (Dissolve 6.0 g of cobaltic nitrate and 40.0 g of ammonium thiocyanate in water and dilute to 200 ml). Stopper and mix well, allow to stand at room temperature for one and a half hours. Shake the mixture every 15 minutes. Allow the mixture to stand for another half an hour before determination. Discard the upper layer. Read the absorbance of the lower layer of dichloromethane at 620 nm by ultraviolet-visible spectrophotometry (Appendix II) using dichloromethane as a blank. Measure accurately 0 μ l, 10 μ l, 25 μ l, 50 μ l, 75 μ l and 100 μ l of polysorbate 80 reference solution (Weigh accurately 100 mg of polysorbate 80, calibrated accurately, and dissolve in small amount of water. Transfer the solution totally to a 100 ml volumetric flask and dilute to volume with water) and put into a series of centrifugal tubes containing 1 ml of water separately. Mix well. To each tube, add accurately 2.0 ml of dichloromethane and 3.0 ml of cobaltic ammonium thiocyanate solution. Stopper and mix well. Carry out the same procedure as that for test sample starting from "allow to stand at room temperature for one and a half hours".

A linear regression equation is obtained by regressing

the concentrations of polysorbate 80 tubes with the corresponding absorbance. The correlation coefficient obtained shall be not less than 0.98. The polysorbate 80 content (μ g/ml) of the test sample is obtained by inserting the absorbance of the test sample into the linear regression equation.

VI I Determination of Residual Glutaraldehyde Content

The method is based on the principle that glutaraldehyde reacts with 2,4-dinitrophenylhydrazine to form n-pentanal-dinitrophenylhydrazine. The glutaraldehyde content of test sample is determined with high performance liquid chromatography (Appendix III B).

Parameters for chromatography

The chromatographic column of 4.6 mm in diameter and 250 mm in length is filled with octadecyl silane of silica gel (SG120) of 5 μ m grain size. The mobile phase is 70% acetonitrile solution at a flow rate of 1.2 ml per minute. Record the chromatogram at 360 nm for 30 minutes.

Procedure

Weigh accurately a quantity of glutaraldehyde and dissolve in water to prepare a glutaraldehyde reference solution at a concentration of 10 μ g per ml. Measure accurately 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of glutaraldehyde reference solution and put into test tubes separately. Make up the volume of each tube to 1.0 ml with water. To each tube, add accurately 1 ml of mobile phase solution and 0.1 ml of 2,4-dinitrophenylhydrazine solution (Dissolve 2.4 g of 2,4-dinitrophenylhydrazine in 30% perchloric acid solution to make a 100 ml solution). Mix by a mixer immediately. Filter with a 0.45 μ m membrane separately. Centrifuge a quantity of test sample at 3000 r/min for 10 minutes. Measure accurately 1 ml of the supernatant and carry out the same procedure as that for CRS starting from "add accurately 1 ml of mobile phase solution". Measure accurately 10 μ l of each of reference solutions and test sample solution, and inject into the apparatus separately. Record the chromatogram.

A linear regression equation is obtained by regressing the concentration of glutaraldehyde reference solutions with the corresponding peak areas. The glutaraldehyde content of test sample is obtained by inserting its peak area into the equation.

[Note]

(1) The purity of glutaraldehyde solution used for the preparation of reference solution has been determined by chromatography and the amount of 0.1 g is calculated on the base of 100% glutaral-

dehyde.

(2) The correlation coefficient of the linear regression equation shall be not less than 0.99.

VI J Determination of Tributylphosphate Content

The residual tributyl phosphate content in test sample is determined by gas chromatography (Appendix III C).

Parameters for chromatography and test for system suitability

Acid denatured polyethylene glycol (20M) capillary column is used. The temperature of column is 140°C and that of vaporizer is 190°C. Flame ionization detector (FID) or nitrogen phosphorus detector is used at a temperature of 210°C. Flow gas is pure nitrogen at a flow rate of 60 ml per minute. Detection parameters may also be chosen according to the instrument used. The number of theoretical plates of column based on the peak of tributyl phosphate shall be not less than 5000. The separation degree between tributyl phosphate and tripropyl phosphate shall be not less than 1.5. The relative standard deviation (RSD) for the peak area ratio of tributyl phosphate to tripropyl phosphate shall be not more than 5% in five successive determinations of tributyl phosphate reference solution.

Preparation of internal standard

Weigh accurately a quantity of tripropyl phosphate and dissolve in n-hexane to prepare volumetrically a solution of 400 µg per ml.

Procedure

Measure accurately 3 ml of test sample and put into a glass centrifugal tube with stopper. Add accurately 50 µl of tripropyl phosphate internal standard solution and 0.75 ml of 1.5 mol/L perchloric acid solution. Oscillate for 1 minute and incubate in 37°C water bath for 10 minutes. Add 4 ml of n-hexane and oscillate for 2 minutes. Centrifuge at 2000 r/min for 20 minutes. Suck out the upper n-hexane layer carefully, concentrate to about 0.2 ml with blowing air (heating is not allowed) and inject 0.1 µl into the gas chromatographic device.

Weigh accurately a quantity of tributyl phosphate CRS and dissolve in n-hexane to prepare volumetrically a solution of 600 µg per ml. To a set of glass centrifugal tubes with stoppers, each containing 3 ml of water, add 10 µl, 20 µl, 40 µl, 60 µl and 80 µl of tributyl phosphate reference solutions (600 µg/ml) separately. To each tube, add accurately 50 µl of tripropyl phosphate internal standard solution. Carry out the same procedure as that for test sample starting from "oscillate for 1 minute".

A regression equation is obtained by regressing the tributyl phosphate concentration of reference solutions with the corresponding peak area ratios of tributyl phosphate reference solutions to the internal standard. Calculate the tributyl phosphate content (µg/ml) of sample solution.

[Note]

(1) The evaporation speeds of test sample solution and reference solution shall be kept in conformity as much as possible. If emulsion fails to disappear completely after centrifugation, oscillate the tube slightly on the oscillator and centrifuge once again.

(2) The correlation coefficient of the linear regression equation shall be not less than 0.99.

VI K Determination of Sodium Caprylate Content

The sodium caprylate content in test sample is determined by gas chromatography (Appendix III C).

Parameters for chromatography and test for system suitability

Acid denatured polyethylene glycol (20M) capillary column is used. The temperature of column is 160°C and that of vaporizer is 230°C. Flame ionization detector (FID) is used at the temperature of 230°C. Flow gas is pure nitrogen at a flow rate of 35 ml per minute. The separation degree between peaks of caprylic acid and heptanoic acid shall be greater than 1.5. The tailing factor for the peak of caprylic acid shall be 0.95-1.20. The relative standard deviation (RSD) for the peak area ratio of caprylic acid to heptanoic acid shall be not greater than 5% in five successive determinations of caprylic acid reference solution.

Preparation of internal standard solution

Dissolve heptanoic acid in trichloromethane to prepare a solution of 10 mg per ml.

Procedure

Dilute accurately a quantity of test sample with water to a protein concentration of 40-50 g/L. Measure accurately 0.5 ml of the diluted sample in a test tube. Add 30 µl of internal standard solution and 0.2 ml of 1.5 mol/L perchloric acid solution. Mix for 1 minute on an oscillator. Add 4 ml of trichloromethane and lid a cover. Mix on an oscillator vigorously for 2 minutes. Centrifuge at 3000 r/min for 20 minutes. Discard the upper water layer and pour the trichloromethane layer into a 10 ml test tube carefully. Evaporate out the trichloromethane until dry. Add accurately 100 µl of trichloromethane to dissolve the residue. Measure accurately 0.1 µl and inject into the gas chromatographic apparatus.

Weigh accurately 0.15 g of caprylic acid and dissolve in a small volume of trichloromethane.

Transfer totally to a 10 ml volumetric flask and dilute to volume with trichloromethane. Measure accurately 10 μ l, 20 μ l, 30 μ l, 40 μ l and 50 μ l of caprylic acid reference solution and put into a set of test tubes separately. To each tube, add accurately 30 μ l of internal standard solution. Mix for 1 minute on an oscillator. Add 4 ml of trichloromethane to each tube and evaporate the trichloromethane until dry. Add accurately 100 μ l of trichloromethane to each tube to dissolve the residues and proceed with the same procedures as above.

A regression equation is obtained by regressing the concentration of caprylic acid reference solution with the corresponding peak area ratios of caprylic acid CRS to the internal standard. Calculate the amount of caprylic acid (A) in test sample solution. The sodium caprylate content in test sample is calculated by the following equation.

$$\text{Sodium caprylate content (mmol/g protein)} = \frac{A \times N}{144.22 \times B \times C \times 1000}$$

Where: A = Amount of caprylic acid in test sample solution, μ g;

B = Volume of test sample solution taken, i. e. 0.5 ml;

N = Dilution factor of test sample;

C = Protein content of test sample, g/ml.

The value 144.22 is the relative molecular weight of caprylic acid.

[Note]

- (1) 1 mmol of caprylic acid is equivalent to 1 mmol of sodium caprylate.
- (2) The evaporation speed of sample solution and reference solution shall be kept in conformity as far as possible.
- (3) The correlation coefficient of the regression equation shall be not less than 0.99.

VI L Determination of Free Formaldehyde Content

The method is based on the principle that fuchsin-sulfurous acid can react with formaldehyde and form a violet complex. The formaldehyde content in test sample is determined by spectrophotometry.

Preparation of reference solution

Measure accurately a quantity of standardized formaldehyde solution and put into a 500 ml volumetric flask. Dilute to volume with water and mix well. A formaldehyde reference stock solution at a concentration of 0.05% is prepared.

Measure accurately 10 ml of 0.05% formaldehyde reference stock solution and put into a 100 ml volumetric flask. Dilute to volume with water and mix well. A formaldehyde reference solution at a concentration of 0.005% is prepared. This solution shall be prepared just before use.

Procedure

A test sample solution is prepared by measuring accurately 1 ml of test sample and dilute to a formaldehyde concentration of about 0.005% with water. Transfer accurately 1 ml of the test sample solution to a 50 ml test tube with stopper. Add 4 ml of water, 10 ml of fuchsin-sulfurous acid solution and 10 ml of mixed acid solution (Put 783 ml of water into a beaker. Add 42 ml of hydrochloric acid and 175 ml of sulfuric acid slowly with gently stirring. Mix well). Mix well, allow to stand for 3 hours at 25°C. Read the absorbance at 590 nm by ultraviolet-visible spectrophotometry (Appendix II A).

Measure accurately 0 ml, 0.5 ml, 1.0 ml, 1.5 ml and 2.0 ml of 0.005% formaldehyde reference solution and put into a series of 50 ml test tubes with stoppers separately, add water to 5 ml. Other procedures are the same as that for test sample solution starting from the addition of 10 ml of fuchsin-sulfurous acid solution.

A linear regression equation is obtained by regressing the concentration of formaldehyde reference solutions with the corresponding absorbance. The concentration of formaldehyde in test sample solution is obtained by inserting its absorbance to the regression equation. Calculate the free formaldehyde content in test sample.

[Note]

- (1) Preparation of fuchsin-sulfurous acid solution and calibration of sulfur dioxide content

Weigh 4.5 g of basic fuchsin and put into a 3000 ml conical flask. Add 1500 ml of water. Dissolve the fuchsin completely by shaking or heating. Cool down. Add 10 g of sodium sulfite and mix well. Allow the mixture to stand for 5-10 minutes. Then, add 40 ml of 3 mol/L sulfuric acid solution and mix well again. Stopper tightly with a rubber stopper, allow to stand overnight. If colour appears, add 5-10 g of bone charcoal and mix rapidly. Filter quickly with a Buchner funnel. The filtrate is fuchsin-sulfurous acid solution. The sulfur dioxide content of this solution may be controlled at a range of 28-48 mol/L. The excess of sulfur dioxide may be expelled by aeration. If the sulfur dioxide content is too low, more sulfur dioxide gas may be conducted into the solution.

Determination of sulfur dioxide content

Measure 10 ml of fuchsin-sulfurous acid solution and put into a conical flask. Add 20 ml of water and 5 ml of starch indicator solution. Titrate with 0.1 mol/L iodine VS until light blue colour appears.

Calculate the sulfur dioxide content according to the following equation:

$$\text{sulfur dioxide content (mmol/L)} = 50 \times V \times C$$

Where: V = Volume of 0.1 mol/L iodine VS consumed, ml;

C = Concentration of iodine VS, mol/L.

- (2) Calibration of formaldehyde solution

Measure accurately about 1.5 ml of formaldehyde, 37% aqueous solution and put into a conical flask. Add 10 ml of water, 25 ml of hydrogen peroxide solution and two drops of bromothymol blue indicator solution. Add 1 mol/L sodium hydroxide VS dropwise until the solution shows a blue colour. Then, add accurately 25 ml of 1 mol/L sodium hydroxide VS. Put a small funnel on top of the conical flask. Heat the flask in a water bath for 15 minutes, shaking constantly. Cool down. Wash the funnel with water and let the washings flow into the conical flask. Add two drops of bromothymol blue indicator solution and titrate with 1 mol/L hydrochloric acid VS until the solution shows a yellow colour. Calibrate the result with a blank test. One ml of 1 mol/L sodium hydroxide VS is equivalent to 30.03 mg of formaldehyde.

(3) Sometimes, the colour developing times of test sample solution and reference solutions are different. In such case, fuchsin-sulfurous acid solution may be added earlier for those develop slower as required.

(4) If the test sample contains phenol red, the standard tubes shall be calibrated correspondingly.

VI M Determination of Phenol Content

The method is based on the principle that phenol combines with bromine, which is produced by the reaction of bromate and hydrochloric acid, and forms tribromophenol. The excess bromine reacts with potassium iodide and iodine is released. The released iodine is then titrated with sodium thiosulfate and the phenol content of test sample can be calculated according to the sodium thiosulfate VS consumed.

Procedure

Transfer accurately 1 ml of test sample to an iodine bottle and add 50 ml of water. Add accurately 15-25 ml (25 ml for the test sample in which phenol content is 0.3% - 0.5% and 15 ml for the test sample in which phenol content is less than 0.3%) of 0.02 mol/L bromine solution (Dissolve 0.56 g of potassium bromate and 3 g of potassium bromide in water and dilute to 1000 ml). Then, add 10 ml of 6 mol/L hydrochloric acid solution along the inner wall of the bottle and stopper tightly. Mix well, allow to stand for 30 minutes in a dark place. Add 2 ml of 25% potassium iodide solution onto the neck of the iodine bottle. Open the stopper slightly to let the solution flow into the bottle. Stopper tightly and shake well. Wash the neck of the bottle with a small volume of water. Titrate with 0.02 mol/L sodium thiosulfate VS until the end point is nearly reached. Add about 0.5 ml of starch indicator solution and continue the

titration until the blue colour disappears. The titration result is calibrated with a blank test.

Result calculation

Phenol content of test sample (%) = $(V_0 - V_1) \times C \times 15.69 \times 100 / 1000$

Where: V_0 = Titer of blank test, ml;

V_1 = Titer of test sample ml;

C = Concentration of sodium thiosulfate VS, mol/L.

The value of 15.69 is the one sixth of the relative molecular weight of phenol.

[Note]

(1) Preparation and calibration of 0.1 mol/L sodium thiosulfate VS

Dissolve 26 g of sodium thiosulfate and 0.20 g of anhydrous sodium carbonate in freshly boiled and cooled water and dilute to 1000 ml. Mix well. Allow the mixture to stand for 1 month and filter.

Weigh accurately 0.15 g of potassium dichromate primary standard, which has been dried to constant weight at 120°C, and put into an iodine bottle. Add 50 ml of water and shake until the solid is dissolved. Add 2.0 g of potassium iodide and dissolve by shaking gently. Add 40 ml of diluted hydrochloric acid (5.7 → 100). Stopper tightly and mix well. Allow the mixture to stand for 10 minutes in a dark place. Add 250 ml of water and titrate with the sodium thiosulfate solution until the end point is nearly reached. Add 3 ml of starch indicator solution (Suspend 0.5 g of soluble starch with 5 ml of water and pour slowly into 100 ml of boiling water with constantly stirring. Continue the boiling for 2 minutes and cool down. Decant and collect the upper clear liquid. The solution shall be prepared just before use). Continue the titration until blue colour disappears and a brilliant green colour appears. Calibrate the titration result with a blank test. One ml of 0.1 mol/L sodium thiosulfate solution is equivalent to 4.903 mg of potassium dichromate. The concentration of the volumetric solution is calculated according to the weight of potassium dichromate taken and the volume of volumetric solution consumed.

(2) Preparation of 0.02 mol/L sodium thiosulfate VS

Measure accurately 100 ml of 0.1 mol/L sodium thiosulfate titration, dilute volumetrically to 500 ml with water and mix well.

(3) A limit test may be carried out.

VI N Determination of Metacresol Content

The method is based on the principle that metacresol reacts with 4-amino antipyrine and potassium ferricyanide under an alkaline condition

and forms a red compound. The metacresol content in test sample is determined by spectrophotometry.

Procedure

A volume of test sample is diluted 50-fold volumetrically to prepare a test sample solution. Mix 1.0 ml of test sample solution with 5.0 ml of water thoroughly. Add successively 1.0 ml of pH 9.8 buffer solution (Dissolve 6.36 g of anhydrous sodium carbonate and 3.36 g of sodium bicarbonate in water and dilute to 800 ml. Adjust pH to 9.8 with 1 mol/L hydrochloric acid solution and dilute to 1000 ml with water), 0.3% 4-amino antipyrine solution, 1.2% potassium ferricyanide solution and 1 mol/L potassium dihydrogen phosphate solution. Mix well, allow to stand for 10 minutes at room temperature, protected from light. Read the absorbance at 510 nm according to ultraviolet-visible spectrophotometry (Appendix II A).

Weigh accurately a quantity of metacresol and dissolve in a small volume of water. Totally transfer to a suitable volumetric flask and dilute with water to prepare a metacresol reference solution at a concentration of 10 µg of metacresol per ml. Measure accurately 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, 5.0 ml and 6.0 ml of metacresol reference solution and put into a series of test tubes separately. To each tube, make up the volume to 6.0 ml with water. Carry out the same procedure as that for test sample solution starting from "add successively 1.0 ml of pH 9.8 buffer solution".

A regression equation is obtained by regressing the concentration of metacresol reference solutions with the corresponding absorbance. The metacresol content (mg/ml) of test sample is obtained by inserting its absorbance into the regression equation.

VI O Determination of Trichloromethane Content

The method is based on the principle that trichloromethane, after extraction with ether, reacts with alkaline pyridine and forms a red compound after being warmed in water bath. The trichloromethane content in test sample is determined by spectrophotometry.

Procedure

A test sample solution is obtained by measuring accurately 1 ml of test sample, put into a 5 ml volumetric flask and dilute to volume with water. Transfer accurately 0.5 ml of the test sample solution to a test tube with stopper and add 18 ml of ether, which is free of peroxide. Shake vigorously for 3 minutes, allow to stand. Transfer accurately 5 ml of the ether extract to a graduated

test tube with stopper. Add 5 ml of alkaline pyridine (Mix two volumes of 20% sodium hydroxide solution with five volumes of pyridine and shake vigorously until the mixture becomes alkaline. Allow the mixture to stand statically for separation. Collect the supernatant. If the colour of pyridine is red, it shall be redistilled. This solution shall be prepared just before use) and mix well. Heat in 80°C water bath for 8 minutes and cool down in cold water. Make up the volume to 12 ml with water. Oscillate sufficiently, allow to stand statically for separation of liquids. Discard the upper layer and measure the absorbance at 520 nm by ultraviolet-visible spectrophotometry (Appendix II A).

Measure accurately 1 ml of trichloromethane and put into a 100 ml volumetric flask. Dilute to volume with ethanol. Just before use, dilute the solution volumetrically to prepare a 0.1% trichloromethane reference solution with water. Measure accurately 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml and 0.5 ml of 0.1% trichloromethane reference solution and put into five test tubes with stoppers separately. Make up the volume of each tube to 0.5 ml with water. Carry out the same procedure as that for test sample solution starting from the addition of 18 ml of peroxide-free ether.

A linear regression equation is obtained by regressing the volume of 0.1% trichloromethane reference solutions with the corresponding absorbance. The content of trichloromethane in test sample is obtained by inserting the absorbance test sample solution into the regression equation.

[Note]

- (1) Sodium hydroxide used for the preparation of alkaline pyridine must be free of sodium carbonate otherwise the solution will be turbid.
- (2) A limit test may be carried out.

VI P Determination of Saccharides and Sugar Alcohol Content in Human Blood Products

The contents of saccharides and sugar alcohol in human blood products are determined with high performance liquid chromatography (Appendix III B).

Parameters for chromatography and test for the suitability of system

Cation chromatographic column (H^+) at a diameter of 7.8 mm and a length of 300 mm filled with co-polymerized phenylethylene and divinylbenzene matrix with a grain size of 8 µm or 9 µm is used. Column temperature is 50°C (20-30°C for sucrose determination) and mobile phase is 0.004 mol/L sulfuric acid solution at a flow rate of 0.8 ml per minute. The detector is a differential

refractometer.

Mix 1 ml of 2% maltose and 1 ml of 1.5% sulfosalicylic acid. Inject 20 μ l of the mixture into the chromatographic column and record the chromatogram. The separation degree between peaks of maltose and sulfosalicylic acid shall be more than 1.5 with a tailing factor of 0.95-1.20 calculated as the peak of maltose.

Preparation of reference solutions

(1) Maltose reference solution

Weigh accurately 1.0 g, 2.0 g and 3.0 g of maltose, previously dried to constant weight under reduced pressure, and transfer totally to three 100 ml volumetric flasks separately. Dilute each of them to volume with water and mix well.

(2) Glucose reference solution

Weigh accurately 0.5 g, 1.0 g and 1.5 g of glucose, previously dried to constant weight under reduced pressure, and transfer totally to three 100 ml volumetric flasks separately. Dilute each of them to volume with water and mix well.

(3) Sorbitol reference solution

Weigh accurately 0.5 g, 1.0 g and 1.5 g of sorbitol, previously dried to constant weight under reduced pressure, and transfer totally to three 100 ml volumetric flasks separately. Dilute each of them to volume with water and mix well.

(4) Sucrose reference solution

Weigh accurately 1.0 g, 2.0 g and 3.0 g of sucrose, previously dried to constant weight under reduced pressure, and transfer totally to three 100 ml volumetric flasks separately. Dilute each of them to volume with water and mix well.

Preparation of test sample solution

Measure accurately 1 ml of test sample. Add 4.0 ml of 1.5% sulfosalicylic acid solution and mix well. Allow the mixture to stand at room temperature for at least 2 hours. Centrifuge at 3000 r/min for 10 minutes and collect the supernatant for use.

Procedure

Measure accurately 20 μ l of reference solutions and test sample solutions and inject into the liquid chromatographic device separately. Record the chromatograms.

Regression equations are obtained by regressing the content of reference solutions (g/L) of individual saccharide or sugar alcohol with the corresponding peak areas. Calculate the content of individual saccharide or sugar alcohol (A) in test sample solution. The saccharide or sugar alcohol content of the test sample is calculated by the following equation:

$$\text{Saccharide or sugar alcohol content (g/L)} = A \times n$$

Where: A = Saccharide or sugar alcohol content in test sample solution, mg;

n = Dilution factor of test sample;

[Note]

(1) The volume of CRS and test samples loaded

may be properly adjusted according to the content of saccharide or sugar alcohol in test sample.

(2) The correlation coefficient of linear regression equation shall be not less than 0.999.

(3) The mobile phase and its flow rate, column temperature for cation chromatographic column (H^+) obtained from different suppliers may be different. The chromatographic parameters may be properly adjusted according to the instructions.

VI Q Determination of Polymer Content in Human Albumin

The polymer content in human albumin is determined with molecular exclusion chromatography (Appendix III D).

Parameters for chromatography and test for the suitability of system

High efficiency volume exclusion chromatographic column (SEC, exclusion limit 300 kD, grain size 10 μ m) at a diameter of 7.5 mm and a length of 30 cm or 60 cm filled with hydrophilic silica gel is used. The mobile phase is 0.2 mol/L pH 7.0 phosphate buffer solution containing 1% isopropanol (Mix 200 ml of 0.5 mol/L sodium dihydrogen phosphate, 420 ml of 0.5 mol/L disodium hydrogen phosphate and 15.5 ml of isopropanol with 914.5 ml of water) at a flow rate of 0.6 ml per minute. The wavelength for detection is 280 nm.

Inject 20 μ l of protein solution at a concentration of 4 mg per ml into the chromatographic column and record the chromatogram. The separation degree between peaks of monomer and dimer shall be more than 1.5. The tailing factor shall be 0.95-1.40 calculated as peak of protein monomer.

Procedure

Dilute a quantity of test sample with mobile phase to a protein concentration of about 4 mg per ml. Inject 20 μ l into the chromatographic column. Record the chromatogram for 30 minutes (chromatographic column length 30 cm) or 60 minutes (chromatographic column length 60 cm).

Calculate the result using area regression method. Divide the content (%) of total exclusion peak by 2 to obtain the polymer content of human albumin.

VI R Determination of IgG Monomer and Dimer in Human Immunoglobulins

The sum of IgG monomer and dimer in human immunoglobulins is determined by molecular exclusion chromatography (Appendix III D).

Parameters for chromatography and test for the

suitability of system

High efficiency volume exclusion chromatographic column (SEC, exclusion limit 300 kD, grain size 10 μm or 13 μm), filled with hydrophilic silica gel, at a diameter of 7.5 mm and a length of 30 cm or 60 cm is used. The mobile phase is 0.2 mol/L phosphate buffer solution (pH 7.0) containing 1% isopropanol (Mix 200 ml of 0.5 mol/L sodium dihydrogen phosphate solution, 420 ml of 0.5 mol/L disodium hydrogen phosphate solution and 15.5 ml of isopropanol with 914.5 ml of water thoroughly) at a flow rate of 0.6 ml per minute. The wavelength for detection is 280 nm.

Inject 20 μl of protein solution at a concentration of 4 mg per ml into the chromatographic column and record the chromatogram. The separation degree between peaks of monomer and dimer shall be more than 1.5. The tailing factor shall be

0.95-1.40 calculated as peak of protein monomer.

Procedure

Dilute a quantity of test sample with mobile phase to a protein concentration of 4 mg per ml and inject 20 μl into the chromatographic column. Record the chromatogram for 60 minutes. Calculate the result using area regression method. The content of monomer and dimer peaks is the sum of monomer and dimer representing the percentage of the total area of the chromatogram. The boundary of peaks in the chromatogram is the vertical line from the point at the gorge between two peaks to the base line. The principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a retention time relative to monomer of about 0.85.

Appendix VII

VII A Determination of Phosphorus Content

The method is based on the principle that organic phosphorus is transformed into inorganic phosphorus. The phosphate radical reacts with ammonium molybdate in acid solution and forms ammonium phosphomolybdate. In the presence of reducing agent, phosphomolybdate is reduced to a blue colour substance called "Molybdenum blue" (A mixture of molybdenum trioxide and molybdenum pentoxide). The phosphorus in test sample is determined by spectrophotometry.

Procedure

Measure accurately a quantity of test sample (containing about 4-20 μg of phosphorus) and put into a test tube. Add four drops (about 0.08 ml) of sulfuric acid and heat until carbonization occurs. Add two drops (about 0.06 ml) of perchloric acid. Digest until the liquid becomes clear and colourless. Allow to stand for a while and add 2 ml of water immediately. Add 0.4 ml of 0.04 mol/L ammonium molybdate solution (Dissolve 5 g of ammonium molybdate in water and dilute to 100 ml with water) and mix well. Then, add 0.2 ml of reducing reagent (Dissolve 6 g of sodium bisulfite, 1.2 g of sodium sulfite and 0.1 g of 1-amino-2-naphthol-4-sulfonic acid in water and dilute to 50 ml. Store in a brown colour bottle and use within a week) and mix well again. Dilute to 6 ml with water. Allow to stand for 15-20 minutes and read the absorbance at 820 nm by ultraviolet-visible spectrophotometry (Appendix II A).

Weigh accurately 439.3 mg of potassium dihydrogen phosphate, which has been dried to constant weight, and dissolve in a small volume of water. Transfer totally to a 100 ml volumetric flask and dilute to volume with water. Measure accurately 2 ml of the above phosphorus solution and put into a 100 ml volumetric flask. Dilute to volume with water to prepare a phosphorus reference solution at a concentration of 20 μg per ml.

Measure accurately 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of phosphorus reference solution and put into a series of test tubes separately. Make up each volume to 1 ml with water. Carry out the

same procedure as that for test sample starting from the addition of sulfuric acid and read the absorbance of each tube.

A linear regression equation is obtained by regressing the volume of phosphorus reference solution with the corresponding absorbance. The volume of test sample is obtained by inserting its absorbance into the regression equation.

Phosphorus content of test sample ($\mu\text{g}/\text{ml}$) = $V \times C_R / V_X$.

Where: V = Volume of phosphorus reference solution corresponding to the absorbance of the test sample, ml;

C_R = Concentration of phosphorus reference solution, $\mu\text{g}/\text{ml}$;

V_X = Volume of test sample, ml.

[Note]

(1) If necessary, one to two drops of 30% hydrogen peroxide may be added during the digestion with perchloric acid but hydrogen peroxide must be decomposed completely at last.

(2) If the mixture after digestion with perchloric acid is cooled down, it shall be heated again before adding water.

(3) To determine the phosphorus content of group A meningococcal polysaccharide vaccine, at least three ampoules of test samples shall be reconstituted and mixed for determination.

VII B Determination of Thimerosal Content

The method is based on the principle that organic mercury compound may be digested by strong acid to form inorganic mercuric ion. After extraction by carbon tetrachloride, the mercuric ion reacts with disulfurazone and forms a green compound. The thimerosal content of test sample can be calculated from the disulfurazone VS consumed.

Reagent

(1) Disulfurazone VS

Weigh accurately 50 mg of disulfurazone and dissolve in a small amount of chloroform. Transfer totally to a 100 ml volumetric flask and dilute to volume with carbon tetrachloride to prepare a disulfurazone stock solution.

Measure accurately 2.5 ml of disulfurazone stock

solution, put into a 100 ml volumetric flask and dilute to volume with carbon tetrachloride just before use. Mix well and a disulfurazone VS is obtained. Store in a dark cold place.

(2) Mercury reference solution

Weigh accurately 0.135 g of mercuric chloride, which has been dried to constant weight in a sulfuric acid desiccator, and dissolve in a small volume of 0.5 mol/L sulfuric acid solution. Transfer totally to a 100 ml volumetric flask and dilute to volume with 0.5 mol/L sulfuric acid solution. Mix well to obtain a mercury reference stock solution.

Upon use, measure accurately a quantity of mercury reference stock solution and put into a 100 ml volumetric flask. Dilute to volume with 0.5 mol/L sulfuric acid solution and mix well. One ml of this reference solution is equivalent to 50 µg of Hg.

Procedure

(1) Digestion

Measure accurately a quantity (containing about 50 µg of mercury) of test sample and put into a 150 ml round bottom flask fitted with a 40 cm long reflux condenser through ground joint. Add 2 ml of sulfuric acid solution and 0.5 ml of 8.0 mol/L nitric acid solution. Mix well and reflux on an electric heater for 15 minutes (or heat in 85-90°C water bath for one hour using a 3 cm×24 cm test tube with stopper). Cool down. Add 40 ml of water and 5 ml of 20% hydroxylamine hydrochloride.

(2) Titration

Wash the digested test sample into a 125 ml separating funnel for several times with water at a total volume of 40 ml and titrate with disulfurazone VS. Add about 2 ml of the volumetric solution each time at the beginning and reduce the volume to 0.5 ml gradually. At last, the volume of volumetric solution may be reduced to 0.2 ml. Shake for 10 seconds and discard the carbon tetrachloride after the separation of liquids by standing following each addition. Continue the titration procedure until the green colour of disulfurazone persists, i. e. the end-point.

(3) Standardization of disulfurazone solution

Measure accurately 1 ml of mercury reference solution and put into a 125 ml separation funnel. Add 2 ml of concentrated sulfuric acid, 80 ml of water and 5 ml of 20% hydroxylamine hydrochloride solution. Carry out the same procedure starting from "titration with disulfurazone VS".

Calculate according to the following equation:

$$\text{Thimerosal content (\%)} = V_1 \times 0.050 \times 2.02 \times 100 / (V_2 \times V_3 \times 1000)$$

Where: V_1 = Titer of test sample, ml;

V_2 = Titer of mercury reference solution, ml;

V_3 = Volume of test sample, ml;

0.050 = Concentration of mercury reference

solution, mg/ml;

The value of 2.02 is a constant (1 g of mercury is equivalent to 2.02 g of thimerosal).

[Note]

(1) A small amount of flocculus, without influence on the test result, may occur during the titration of test sample of antitoxins or immunoglobulins by water bath digestion method.

(2) A limit test may be carried out.

VII C Determination of Ammonium Sulfate Content

The method is based on the principle that ammonium sulfate may be decomposed by sodium hydroxide and ammonia is released. The released ammonia is then absorbed by boric acid to form ammonium borate, which may be titrated with acid VS. The ammonium content of test sample can be calculated according to the volume of acid VS consumed.

Preparation of test sample solution

The method for deproteinization is the same as that for the determination of protein content (Appendix VI B, method 1).

Procedure

Measure accurately 10 ml of deproteinized filtrate and put into a Kjeldahl distillator. Add 1 ml of 4% sodium hydroxide solution and a small volume of water. Distill and titrate by semi-micro Kjeldahl method (Appendix VI A). Calibrate the titration result with a blank test.

Calculate according to the following equation:

$$\text{Ammonium sulfate content (\%)} = (V_1 - V_2) \times C \times 14.01 \times 4.715 \times 2 \times 100 / 1000$$

Where: V_1 = Titer of sample, ml;

V_2 = Titer of blank control, ml;

C = Concentration of sulfuric acid VS, mol/L;

The value of 4.715 is a constant (1 g of nitrogen is equivalent to 4.715 g of ammonium sulfate).

The value of 14.01 is the relative atomic weight of nitrogen.

VII D Determination of Moisture Content

(Karl Fischer's Method)

A. Volumetric titration

This method is based on the quantitative reaction of water with a solution of sulfur dioxide and iodine in pyridine and methanol. The apparatus used should be dry and moistureproof. The determination of water is preferably carried out in a

low humidity environment.

(1) *Preparation of Karl Fischer reagent* Place 110 g of iodine, previously dried in a desiccator over sulfuric acid for more than 48 hours, in a dry stoppered conical beaker, add 160 ml of anhydrous pyridine and cool, shake to effect complete dissolution. Add 300 ml of anhydrous methanol and weigh the conical beaker. Keep the conical beaker cold in an ice bath and keep the atmospheric moisture excluded from the system, pass dry sulfur dioxide into the conical beaker until the increase of weight is 72 g, add anhydrous methanol to produce 1000 ml. Stopper tightly and mix well, allow to stand for 24 hours protected from light.

This reagent should be preserved in tightly closed containers, protected from light and stored in a cool and dry place, standardized before use.

(2) *Standardization of Karl Fischer reagent* Standardize with a water determination apparatus directly or place about 30 mg of redistilled water, accurately weighed, in a dry stoppered flask, add 2-5 ml of anhydrous methanol. Titrate with Karl Fischer reagent until the colour changes from pale yellow to reddish brown. The end-point may also be determined electrometrically by dead-stop titration (Appendix VII A of Volume II). Perform a blank titration and calculate the water equivalent of the reagent in mg of water per ml by the formula:

$$F = \frac{W}{A - B}$$

in which F is the water equivalent of the reagent, W is the weight of redistilled water in mg, A is the volume, in ml, of the reagent consumed in the titration of water and B is the volume, in ml, of the reagent consumed in the blank titration.

(3) *Procedure* Weigh accurately a quantity of the substance being examined which is anticipated to consume 1-5 ml of Karl Fischer reagent and determine with a water determination apparatus directly or place in a dry stoppered flask, add 2-5 ml of anhydrous methanol, titrate with Karl Fischer reagent with continuous shaking or stirring until the colour changes from pale yellow to reddish brown. The end-point may also be determined electrometrically by dead-stop titration (Appendix VII A of Volume II). Perform a blank titration and calculate the percentage of water in the substance being examined by the formula:

$$\frac{(A-B)F}{W} \times 100$$

in which A and B are the volumes in ml of the reagent consumed in the titration of water in the substance being examined and in the blank titration respectively; F is the water equivalent to the reagent in mg per ml and W is the weight of the substance being examined in mg.

B. Coulometric titration

This method is also based on the Karl Fischer reaction and using dead-stop titration (Appendix VII A of Volume II) to determine the content of water. However, compared with volumetric titration, iodine is not added in the form of a volumetric solution from the titration tube but is produced in an iodide-containing solution by anodic oxidation. When all the water has been consumed an excess of iodine occurs, which can make the platinum electrodes polarize, thus indicating the endpoint. According to Faraday's law, the amount of iodine produced is directly proportional to the electric quantity passed through, so the total amount of water can be determined by measuring the total amount of electric quantity consumed. This method is predominantly used for substances with a very low water content (0.1% to 0.0001%). It is particularly suited to chemically inert substances like hydrocarbons, alcohols, and ethers. All the apparatus used must be dry and atmospheric moisture is excluded from the system. Procedure should be operated in a dry place.

Karl Fischer reagent Prepare or purchase the titration reagent according to the requirements of Karl Fischer's coulometric titrator. Calibration of the instrument is not necessary.

Procedure Moisture is eliminated from the system by preelectrolysis, transfer quickly a quantity of test specimen estimated to contain 0.5-5 mg of water, accurately measured, into the anolyte solution. Perform coulometric titration to the electrometric endpoint. Read the water content of the specimen directly from the instrument's display, and calculate the percentage of water in the substance. One mg of water is equivalent to 10.72 Coulomb of electric quantity.

VII E Determination of Sodium Bisulfite Content

The method is based on the principle that sodium bisulfite may react with an excess of iodine. The surplus iodine is then titrated with sodium thiosulfate VS. The sodium bisulfite content of test sample can be calculated according to the volume of sodium thiosulfate VS consumed.

Procedure

Measure accurately a quantity of test sample (containing about 2.5 mg of sodium bisulfite) and put into an iodine bottle. Add accurately 20 ml of 0.1 mol/L iodine solution (Dissolve 36 g of potassium iodide and 13.0 g of iodine in 50 ml of water successively. Add three drops of hydrochloric acid and dilute to 1000 ml with water. Mix well and filter with sintered-glass filter), allow to stand for 5 minutes. Add 2.0 ml of hydrochloric acid (5→10) along the inside wall of the bottle

and mix well. Titrate with 0.1 mol/L sodium thiosulfate VS until the end point is nearly reached. Add 0.5 ml of 0.5% starch indicator solution and continue the titration until the disappearance of blue colour. Calibrate the titration result with a blank test.

Calculate according the following equation:

$$\text{Sodium bisulfite content (\%)} = (V_0 - V_1) \times C \times 5.203 \times 100 / (V_2 \times 1000)$$

Where: V_0 = Titer of blank test, ml;
 V_1 = Titer of test sample, ml;
 V_2 = Volume of test sample, ml;
 C = Concentration of sodium thiosulfate VS, mol/L.

[Note]

Preparation and titration of 0.1 mol/L sodium thiosulfate VS

Dissolve 26 g of sodium thiosulfate and 0.20 g of anhydrous sodium carbonate in freshly boiled and cooled water and dilute to 1000 ml, allow to stand for one month and filter.

Weigh accurately 0.15 g of potassium dichromate primary standard, which has been dried to constant weight, and put into an iodine bottle containing 50 ml of water for dissolution. Add 2.0 g of potassium iodide and dissolve by shaking gently. Add 40 ml of diluted hydrochloric acid (5.7 → 100). Stopper the bottle tightly and mix well. Allow the mixture to stand in a dark place for 10 minutes. Add 250 ml of water and titrate with the sodium thiosulfate solution until the end point is nearly reached. Add 3 ml of starch indicator solution (Suspend 0.5 g of soluble starch in 5 ml of water and pour slowly into 100 ml of boiling water with constantly stirring. Continue the boiling for 2 minutes and cool down. Decant and collect the upper clear liquid. The solution shall be prepared just before use) and continue the titration until the blue colour disappears and a brilliant green colour appears. Calibrate the titration result with a blank test. One ml of sodium thiosulfate VS is equivalent to 4.903 g of potassium dichromate. Calculate the concentration of sodium thiosulfate VS according to the weight of potassium dichromate taken and the volume of the sodium thiosulfate solution consumed.

VII F Determination of Aluminium Hydroxide (or Aluminium Phosphate) Content

The method is based on the principle that aluminium ion reacts with an excess of sodium ethylenediamine tetraacetate and the surplus sodium ethylenediamine tetraacetate is titrated with zinc VS. The aluminium hydroxide (or aluminium phosphate) content in test sample can

be calculated according to the volume of zinc VS consumed.

Procedure

Measure accurately a quantity of test sample (containing about 1-10 mg of aluminium) and transfer to a 250 ml conical flask. Dissolve completely by adding 1.5 ml of phosphoric acid solution (6 → 100). Warm in a water bath if necessary (an additional volume of phosphoric acid solution may be added to the test samples which is not easy to dissolve). Add accurately 10 ml of 0.05 mol/L sodium ethylenediamine tetraacetate VS. Then, add 10 ml of pH 4.5 ammonium acetate buffer solution (Dissolve 7.7 g of ammonium acetate in 50 ml of water. Add 6 ml of glacial acetic acid and dilute to 100 ml with water). Heat in boiling water bath for 10 minutes. Take out the flask from the water bath and cool down to room temperature. Add 1 ml of 0.1% dicresol (xylenol) orange indicator solution. Titrate with 0.025 mol/L zinc VS to the end point when the colour of solution changes from brilliant yellow to orange. Calibrate the titration result with a blank test.

Calculate according to the following equation:

$$\text{Aluminium hydroxide content (mg/ml)} = (V_0 - V_1) \times C \times 78.01 / V_2$$

$$\text{Aluminium phosphate content (mg/ml)} = (V_0 - V_1) \times C \times 121.95 / V_2$$

$$\text{Aluminium content (mg/ml)} = (V_0 - V_1) \times C \times 26.98 / V_2$$

Where: V_0 = Titer of blank test, ml;
 V_1 = Titer of test sample, ml;
 C = Concentration of zinc VS, mol/L;
 V_2 = Volume of test sample, ml;

The values of 78.01, 121.95 and 26.98 are the relative molecular (atomic) weights of aluminium hydroxide, aluminium phosphate and aluminium respectively.

[Note]

(1) Preparation and titration of 0.05 mol/L zinc VS

Weigh 15 g of zinc sulfate (equivalent to about 3.3 g of zinc) and mix with 10 ml of diluted hydrochloric acid (23.4 → 100). Dissolve in a quantity of water and dilute to 1000 ml with water. Mix well. Measure accurately 25 ml of the solution. Add one drop of 0.025% methyl red ethanol solution. Add ammonia test solution dropwise until the solution becomes slightly yellow. Add 25 ml of water, 10 ml of ammonia-ammonium chloride buffer solution (pH 10.0) and a small amount of eriochrome black T indicator solution. Titrate with 0.05 mol/L sodium ethylenediamine tetraacetate VS until the colour changes from purple to pure blue. Calibrate the titration result with a blank test. The concentration of the zinc solution is calculated according to the volume of 0.05 mol/L sodium ethylenediamine tetraacetate VS consumed.

(2) Preparation of 0.025 mol/L zinc VS

Measure accurately 100 ml of 0.05 mol/L zinc VS and dilute volumetrically to 200 ml with water.

(3) Preparation and titration of 0.05 mol/L sodium ethylenediamine tetraacetate VS

Dissolve 19 g of sodium ethylenediamine tetraacetate in water and dilute to 1000 ml. Mix well. Weigh accurately 0.12 g of zinc oxide primary standard, which has been ignited to constant weight at about 800°C. Add 3 ml of diluted hydrochloric acid (23.4 → 100) to dissolve the zinc oxide. Add 25 ml of water and one drop of 0.025% methyl red ethanol solution. Add ammonia test solution dropwise until the colour of solution becomes slightly yellow. Add 25 ml of water, 10 ml of ammonia-ammonium chloride (pH 10.0) buffer solution and a small amount of eriochrome black T indicator solution. Titrate with 0.05 mol/L sodium ethylenediamine tetraacetate VS until the colour of the solution changes from purple to pure blue. Calibrate the titration result with a blank test. One ml of 0.05 mol/L sodium ethylenediamine tetraacetate VS is equivalent to 4.069 mg of zinc oxide. Calculate the concentration of the volumetric solution according to the weight of zinc oxide taken and the volume of the volumetric solution consumed.

Sodium chloride content (g/L) =

$$(V_0 - V_x) \times C \times 58.45$$

Where: V_0 = Titer of blank test, ml;

V_x = Titer of test sample, ml;

C = Concentration of ammonium thiocyanate VS, mol/L;

The value of 58.45 is the relative molecular weight of sodium chloride.

[Note]

(1) Preparation and titration of 0.1 mol/L ammonium thiocyanate VS

Dissolve 8.0 g of ammonium thiocyanate in water and dilute to 1000 ml. Mix well. Measure accurately 25 ml of 0.1 mol/L silver nitrate VS. Add 50 ml of water, 2 ml of nitric acid and 2 ml of 8% ammonium ferric sulfate indicator solution. Titrate with the ammonium thiocyanate solution until the colour of solution becomes faint brownish red. The end point is reached when the colour persists after shaking vigorously. The concentration of the ammonium thiocyanate VS is calculated according to the volume of the volumetric solution consumed.

(2) Preparation of 0.05 mol/L ammonium thiocyanate VS

Measure accurately 100 ml of 0.1 mol/L ammonium thiocyanate VS and dilute volumetrically to 200 ml with water. Mix well.

VII G Determination of Sodium Chloride Content

The method is based on the principle that sodium chloride reacts with an excess of silver nitrate after the proteins in test sample are destroyed by nitric acid. Chloride ion reacts completely with silver nitrate and is precipitated out as silver chloride. The surplus silver nitrate is then titrated with ammonium thiocyanate VS. The sodium chloride content can be calculated according to the volume of ammonium thiocyanate VS consumed.

Procedure

Measure accurately 1 ml of test sample and add accurately 5 ml of 0.1 mol/L silver nitrate solution (Dissolve 17.0 g of silver nitrate in water and dilute to 1000 ml) and mix well. If the protein content of test sample is high, add 2 ml of saturated potassium permanganate solution. Mix well and add 10 ml of 8.0 mol/L nitric acid solution. Digest by heating until the solution becomes clear. Cool down. Add 50 ml of water and 1 ml of 8% ferric ammonium sulfate indicator solution. Titrate with 0.05 mol/L ammonium thiocyanate VS until a faint brownish red colour appears. The end point is reached whenever the colour persists after shaking. Calibrate the titration result with a blank test (digestion may be omitted).

Calculate according to the following equation:

VII H Determination of Citrate Content

Method 1 Colorimetric method**Preparation of sodium citrate reference solution**

Weigh accurately 0.6 g of sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), which has been dried to constant weight under reduced pressure, and dissolve in a small volume of water. Transfer totally to a 100 ml volumetric flask and dilute to volume with water. Mix well. Measure accurately 5 ml of the solution and put into a 50 ml volumetric flask. Dilute to volume with 5% trichloroacetic acid and mix well.

Preparation of test sample solution

Measure accurately 0.5 ml of test sample. Add 4.5 ml of water and 5 ml of 10% trichloroacetic acid. Mix well and heat in 60°C water bath for 5 minutes. Centrifuge at 4000 r/min for 20 minutes and collect the supernatant for use.

Procedure

Measure accurately 1 ml of the test sample solution and put into a 25 ml glass test tube with glass stopper. Add accurately 1.3 ml of pyridine and mix well. Then, add accurately 5.7 ml of acetic anhydride. Mix well immediately and put the tube into 31°C ± 1°C water bath for 35 minutes. Read the absorbance at 425 nm by ultraviolet-visible

spectrophotometry (Appendix II A).

Measure accurately 0.25 ml, 0.5 ml, 0.75 ml and 1.00 ml of sodium citrate reference solution and put separately into a series of 25 ml glass test tubes with stoppers. Add accurately 0.75 ml, 0.50 ml, 0.25 ml and 0.00 ml of 5% trichloroacetic acid respectively (The corresponding citrate concentrations of these mixtures are 0.5 mmol/L, 1.0 mmol/L, 1.5 mmol/L and 2.0 mmol/L respectively). The same procedures as mentioned above are carried out starting from "add accurately 1.3 ml of pyridine".

A linear regression equation is obtained by regressing the concentration of citrate reference solution with the corresponding absorbance. Calculate the citrate concentration of the test sample solution and multiply the result by dilution factor (20), and the concentration of the test sample in mmol/L is obtained.

Method 2 HPLC method

The citric content is determined with high performance liquid chromatography (Appendix III B).

Parameters of chromatography

Ion exchange chromatograph column (H^+) at an inner diameter of 7.8 mm and a length of 300 mm is used. The column is packed with a co-polymer of styrene and divinylbenzene at a grain size of 9 μm or 8 μm . The column temperature is 50°C. The mobile phase is 0.004 mol/L sulfuric acid at a flow rate of 0.8 ml per minute and the detector is differential refractometer.

Procedure

Weigh accurately 0.735 g of sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), which has been dried to constant weight under reduced pressure, and dissolve in a small volume of water. Transfer totally to a 100 ml volumetric flask and dilute to volume with water. Measure accurately 5.0 ml, 10.0 ml and 15.0 ml of the sodium citrate solution and transfer to three 25 ml volumetric flasks separately. Dilute to volume with water to prepare three citrate reference solutions at the concentrations of 5.0 mmol/L, 10.0 mmol/L and 15.0 mmol/L respectively. Measure accurately 20 μl of each of the three tubes of reference solution and inject into the chromatographic column respectively. Record the chromatograms.

Measure accurately 1 ml of test sample solution and put into a 15 ml centrifugal tube. Add accurately 1 ml of 1.5% sulfosalicylic acid solution and mix well. Centrifuge at 2000 r/min for 10 minutes at room temperature. Determine the supernatant by the same method as above.

A linear equation is obtained by regressing the concentration of citrate reference solutions with the corresponding peak areas. Calculate the sodium citrate concentration (mmol/L) of the test sample solution. The citrate concentration (mmol/L) of the test sample is obtained by multiplying the

result by the dilution factor (2) of test sample.

[Note]

(1) The concentration of citrate reference solutions may be adjusted according to the citrate concentration of test sample.

(2) The correlation coefficient of the linear regression shall be not less than 0.999.

(3) The mobile phase and its flow rate, column temperature for cation chromatographic column (H^+) of different producers may be different. The operation parameters can be properly adjusted according to the instructions.

VII I Determination of Potassium Content

The potassium content in test sample is determined with flame photometry.

Procedure

Measure accurately 2 ml of test sample and put into a 50 ml volumetric flask. Dilute to volume with water to prepare a test sample solution. Read the intensity of the light emitted from test sample solution at 769 nm by flame photometry (Appendix II D). Weigh accurately 56.0 mg of potassium chloride, which has been dried to constant weight at 110°C, and dissolve with a quantity of water. Transfer totally to a 500 ml volumetric flask and dilute to volume with water. Measure accurately 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml and 5.0 ml of the above solution and put into a series of 50 ml volumetric flasks separately. Dilute to volume with water to prepare a series of potassium reference solutions at concentrations of 0.03 mmol/L, 0.06 mmol/L, 0.09 mmol/L, 0.12 mmol/L and 0.15 mmol/L respectively. Carry out the test with the same procedures as that for test sample solution.

A linear regression equation is obtained by regressing the concentration of potassium reference solutions with the corresponding intensity of emitted light. The potassium concentration of test sample solution in mmol/L is obtained by inserting its intensity of emitted light into the linear regression equation. Calculate the potassium content of test sample by multiplying the result with the dilution factor (25) of test sample.

VII J Determination of Sodium Content

The sodium content in test sample is determined with flame photometry.

Procedure

Measure accurately 0.5 ml of test sample and put into a 50 ml volumetric flask. Dilute to volume with water to prepare a test sample solution. Read the intensity of light emitted from test sample solution at 589 nm by flame photometry (Appendix II D). Weigh accurately 0.293 mg of sodium chloride, which has been dried to constant weight at 110°C, and dissolve in a small volume of water. Transfer totally into a 100 ml volumetric flask and dilute to volume with water. Measure accurately 0.9 ml, 1.1 ml, 1.3 ml, 1.5 ml and 1.7 ml of the above solution and put into a series of 50 ml volumetric flasks separately. Dilute to volume with water to prepare a series of sodium reference solutions at concentrations of 0.9 mmol/L, 1.1 mmol/L, 1.3 mmol/L, 1.5 mmol/L and 1.7 mmol/L respectively. Carry out the test with the same procedure as that for test sample solution.

A linear regression equation is obtained by regressing the concentration of sodium reference solutions with the corresponding intensity of emitted light. The sodium concentration of the test sample solution in mmol/L is obtained by inserting its intensity of emitted light into the linear regression equation. Calculate the sodium content of the test sample by multiplying the result by the dilution factor (100) of test sample.

VII K Determination of Residual Aluminium Content in Human Albumin

The residual aluminium content in human albumin is determined with atomic absorption spectrophotometry.

Procedure

Prepare blank control solution, test sample solution, and mixed solution of aluminium CRS plus test sample according to the requirements in Table 1. The test sample and 100 ng/ml aluminium reference solution (Measure accurately 0.1 ml of 100 µg/ml aluminium reference solution and put into a 100 ml volumetric flask and dilute to volume with 0.15 mol/L nitric acid) shall be measured accurately. Carry out the determination by atomic absorption spectrophotometry (Appendix II A) at 309.3 nm using aluminium lamp with a slit of 0.7 nm. The temperature controlling program for the drying, incinerating and atomizing of graphite furnace is listed in Table 2. Measure accurately 30 µl each of blank control solution, test sample solution and mixed solution of aluminium CRS and test sample, and inject into the instrument separately. Record the readings and calculate by the following equation:

$$\text{Aluminium content of test sample } (\mu\text{g/L}) = 20 \times (S_0 - B) \times 12.5 / (S - S_0)$$

Where: B = Reading of blank control solution;

S_0 = Reading of test sample solution;

S = Reading of mixed solution of aluminium CRS and test sample.

The value of 20 is the concentration of aluminium in mixed solution of aluminium CRS and test sample, µg/L;

The value of 12.5 is the dilution factor of test sample.

Table 1 Preparation of blank control solution, test sample solution and mixed aluminium reference and test sample solution

Solution	Blank control (ml)	Test sample (ml)	Aluminium CRS plus test sample (ml)
Test sample	—	0.2	0.2
Aluminium CRS (100 ng/ml)	—	—	0.5
0.15 mol/L HNO ₃	2.5	2.3	1.8

Table 2 Temperature-controlling Program for Graphite Furnace

Program	Procedure	Temperature (°C)	Time (second) for Climbing + Duration
1	Pre-heating	80	0+10
2	Drying	220	120+5
3	Incinerating	1200	10+20
4	Atomizing	2600	0+5
5	Clearance	2650	0+5

[Note]

(1) The amounts of test sample and aluminium CRS taken may be adjusted according to the function of instrument used in order to make all the readings fall into the accurate range.

(2) The temperature-controlling program for furnace given in Table 2 may be properly adjusted according to the function of instrument used.

(3) The use of glass containers shall be avoided as much as possible.

VII L Determination of Loss on Drying

Mix the substance being examined thoroughly, if it is in the form of large crystals, reduce them to a size of about 2 mm by crushing. Place about 1 g or the amount specified under individual monographs of the substance being examined in a tared, shallow weighing bottle, previously dried to constant weight at 105°C, unless otherwise directed.

The substance being examined should be evenly distributed to form a layer of not more than 5 mm in thickness, or not more than 10 mm in the case of bulky material. When the loaded bottle is placed in the drying chamber of desiccator, remove the stopper and put it beside the bottle, or leave it

on the bottle in half open position. Upon the opening of the drying chamber or desiccator, the bottle should be closed promptly. If the substance is dried by heating, allow it to cool to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than the specified drying temperature, maintain the bottle with its content below the melting temperature until most of the water is removed, then dry it under the specified conditions.

If a vacuum desiccator or constant temperature vacuum desiccator is to be used, a pressure of 2.67 kPa (20 mmHg) or less should be maintained unless otherwise directed. The desiccants used in a desiccator are usually anhydrous calcium chloride, silica gel or phosphorus pentoxide. Phosphorus pentoxide is often used in a constant temperature vacuum desiccator at 60°C, unless otherwise specified. The desiccants should be kept fully effective.

liquid components of test sample at a certain temperature. The content of total solid is calculated from the residual solid.

Procedure

Method 1 Drying at 105°C

Measure accurately a quantity of test sample and put into a suitable weighing bottle, which has been dried to constant weight. Dry to constant weight in an oven at temperature 105°C.

Method 2 Drying at 50°C

Measure accurately a quantity of test sample and put into a suitable weighing bottle, which has been dried to constant weight. Dry to constant weight in an oven at temperature 50°C.

Calculate according to the following equation:

Total solid of test sample, % (g/ml) = $W \times 100 / V$

Where: W = Constant weight of test sample after drying, g;

V = Volume of test sample, ml.

VII M Determination of Total Solid

The method is based on the evaporation of the

Appendix VIII

VIII A Immunoblot

The antigenic specificity of test sample is detected by the colour developed through enzymological reaction after the test sample is bound to a specific antibody, which is further combined with an enzyme-labelled antibody.

Reagent

(1) TG buffer solution: Dissolve 15.12 g of trishydroxy-methylaminomethane (THAM) and 72 g of glycine in water and dilute to 500 ml. Store at 4°C.

(2) EBM buffer solution: Dilute 20 ml of TG buffer solution and 40 ml of methanol to 200 ml with water. Store at 4°C.

(3) TTBS buffer solution: Dissolve 6.05 g of THAM and 4.5 g of sodium chloride in a quantity of water. Add 0.55 ml of polysorbate 80. Adjust pH to 7.5 with hydrochloric acid and dilute to 500 ml with water. Store at 4°C.

(4) Substrate buffer solution: Dissolve 15 mg of 3, 3'-diaminobenzidine hydrochloride in 25 ml of TTBS buffer solution. Add 5 ml of methanol and 15 µl of 30% hydrogen peroxide solution. Prepare just before use.

Procedure

Comply with the requirements for SDS-PAGE (Appendix IV C). Both the amounts of test sample and reference substance loaded shall be more than 100 ng. Take out the gel and cut off its edge. Immerse the gel into EBM buffer solution for 30 minutes. Immerse six pieces of thick filter paper and one piece of nitro cellulose membrane with EBM buffer solution sufficiently. Both the filter paper and nitro cellulose membrane are the same size of the gel. Transfer the test sample onto the nitro cellulose membrane using semi-dry gel transfer apparatus according to the following procedures: Put three pieces of wet filter paper, nitro cellulose membrane, electrophoresis gel and the other three pieces of wet filter paper in turn onto an electrode plate. Cover with another electrode plate and transfer the test sample for 45 minutes at a constant current of 0.8 mA per cm² of nitro cellulose membrane.

Take the nitro cellulose membrane out and

immerse into blocking solution (TTBS buffer solution containing 10% calf serum or other suitable blocking solutions) to block for 60 minutes. Discard the blocking solution. Add 10 ml of TTBS buffer solution, and then add a quantity of antibody (diluted following the instructions) against test sample, allow to stand overnight at room temperature with shaking. Rinse the nitro cellulose membrane once with TTBS buffer solution, and then wash 3 times each by immersing with the buffer solution for 8 minutes. Discard the buffer solution, add another 10 ml of TTBS buffer solution, then add a quantity of the biotin-labelled secondary antibody, allow to stand at room temperature for 40 minutes with shaking. Rinse the nitro cellulose membrane once with TTBS buffer solution and wash 3 times each by immersing with the buffer solution for 8 minutes. Discard the buffer solution, add another 10 ml of TTBS buffer solution, then add a quantity of avidin solution and biotin-labelled horseradish peroxidase solution, allow to stand at room temperature for 60 minutes with shaking. Rinse the cellulose nitrate membrane once with TTBS buffer solution and wash 4 times each by immersing with the buffer solution for 8 minutes. Discard the buffer solution, add a quantity of substrate buffer solution, allow to stand at room temperature for colour development, protected from light. When a suitable colour appears, stop the reaction by washing with water.

Result evaluation

The result is judged as positive if obvious colour bands appear. If no colour appears, the result is judged as negative.

VIII B Immunodot

The antigenic specificity of test sample is detected by the colour developed through enzymological reaction after the test sample is bound to a specific antibody, which is further combined with an enzyme-labelled antibody.

Reagent

(1) TG buffer solution: Weigh accurately 15.12 g of trishydroxymethylaminomethane (THAM) and 72 g of glycine, dissolve in water and dilute to

500 ml. Store at 4°C.

(2) EBM buffer solution; Dilute 20 ml of TG buffer solution and 40 ml of methanol to 200 ml with water. Store at 4°C.

(3) TTBS buffer solution; Dissolve 6.05 g of THAM and 4.5 g of sodium chloride in a quantity of water. Add 0.55 ml of polysorbate 80. Adjust pH to 7.5 with hydrochloric acid and dilute to 500 ml with water. Store at 4°C.

(4) Substrate buffer solution; Dissolve 15 mg of 3, 3'-diaminobenzidine hydrochloride in 25 ml of TTBS buffer solution. Add 5 ml of methanol and 15 μ l of 30% hydrogen peroxide solution. Prepare just before use.

Procedure

Immerse a piece of nitro cellulose membrane in EBM buffer solution for 15 minutes. Load test sample, negative control (an equal volume of human albumin may be used) and reference substance onto the membrane by dotting. The amount of each sample or control loaded shall be more than 10 ng. Dry the nitro cellulose membrane at room temperature for 60 minutes and immerse in blocking solution (TTBS buffer solution containing 10% calf serum or other suitable blocking solutions) to block for 60 minutes. Discard the blocking solution. Add 10 ml of TTBS buffer solution, then add a quantity of antibody (diluted following the instructions) against test sample, allow to stand overnight at room temperature with shaking. Rinse the nitro cellulose membrane once with TTBS buffer solution and wash 3 times each by immersing with the buffer solution for 8 minutes. Discard the buffer solution, add another 10 ml of TTBS buffer solution, then add a quantity of the biotin-labelled secondary antibody, allow to stand at room temperature for 40 minutes with shaking. Rinse the nitro cellulose membrane once with TTBS buffer solution and wash 3 times each by immersing with the buffer solution for 8 minutes. Discard the buffer solution, add another 10 ml of TTBS buffer solution, then add a quantity of avidin solution and biotin-labelled horseradish peroxidase solution, allow to stand at room temperature for 60 minutes with shaking. Rinse the nitro cellulose membrane once with TTBS buffer solution and wash 4 times each by immersing with the buffer solution for 8 minutes. Discard the buffer solution, add a quantity of substrate buffer solution, allow to stand at room temperature for colour development, protected from light. When a suitable colour appears, stop the reaction by washing with water.

Result evaluation

The result is judged as positive if obvious colour bands appear. If no colour appears, the result is judged as negative.

VIII C Double Immunodiffusion

The specificity of test sample is examined by the formation of precipitation lines between antigen and antibody on agarose plate. Antigen and antibody are added to neighboring wells dug on agarose plate respectively. Precipitation lines of immunocomplex are formed in a certain length of time when the antibody is specific to the antigen, and they are at proper concentrations and with a proper ratio.

Preparation of test sample solution

Dilute test sample with physiological saline to a suitable protein concentration.

Reagent

(1) 0.5% amino black staining solution; Dissolve 0.5 g of amino black 10B in a mixture of 50 ml of methanol, 10 ml of glacial acetic acid and 40 ml of water.

(2) Destaining solution; Mix 45 ml of ethanol, 5 ml of glacial acetic acid and 50 ml of water.

Procedure

Pour the completely swollen 1.5% agarose solution onto a level glass plate (0.19 ml of agarose per cm^2). After the agarose is coagulated, dig several wells each at a diameter of 3 mm in a square array. The distance between neighboring wells is 3 mm (see the following figure). The number of square array is determined according to the actual need. Add antibody into the central well and the test sample solutions into the surrounding wells, except one well is reserved for the corresponding reference serum. The volume of test sample or control added into each well is 20 μ l. Put the agarose plate into a level wet box at 37°C and diffuse for 24 hours. Immerse the agarose plate sufficiently with physiological saline to remove the unbound protein. Stain the plate in 0.5% amino black solution, then decolourize with destaining solution until the background is colourless and clear blue precipitation lines appear. Preserve by a suitable method or copy the profile.

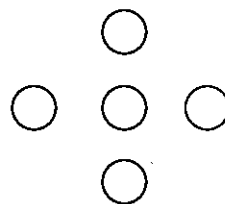


Fig. Square array

Result evaluation

The test is valid if the reference substance forms a corresponding precipitation line. If a precipitation line forms between the test sample and antibody wells, the test sample is judged as corresponding

to the antibody.

VIII D Immunoelectrophoresis

The test sample is separated into antigen bands through electrophoresis and subject to double immunodiffusion with the corresponding antibodies. When the ratio of antigens to antibodies is appropriate, visible precipitation arcs are formed. The components and properties of test sample are analyzed by comparing the sites and shapes of the precipitation arcs with those formed by standard antigens and antibodies.

Reagent

(1) Barbitol buffer solution (pH8.6): Dissolve 4.14 g of barbitol and 23.18 g of barbitol sodium in a quantity of water by heating. Cool down to room temperature. Add 0.15 g of sodium azide and dilute with water to make 1500 ml.

(2) 0.5% amino black staining solution: Dissolve 0.5 g of amino black 10B in a mixture of 50 ml of methanol, 10 ml of glacial acetic acid and 40 ml of water.

(3) 1.5% agarose solution: To 1.5 g of agarose, add 50 ml of water and 50 ml of barbitol buffer solution. Heat to swell completely.

(4) Destaining solution: Mix 45 ml of ethanol and 5 ml of glacial acetic acid with 50 ml of water.

(5) Bromphenol blue indicator solution: Dissolve 50 mg of bromphenol blue in water to make 100 ml.

Reference substance

Normal human serum or other suitable reference substances.

Preparation of test sample solution

Dilute the test sample with physiological saline to a protein concentration of 0.5%.

Procedure

Pour 1.5% agarose solution onto a level glass plate to yield a layer of about 3 mm thick. Coagulate the agarose on standing to form an even thin layer without bubble. Dig two wells, each at a diameter of 3 mm and 10-15 mm apart, at the site about one-third of the full length apart from the negative pole of the plate. Add 10 μ l of test sample and one drop of bromphenol blue indicator solution into the test well, and 10 μ l of normal human serum and one drop of bromphenol blue indicator solution into the control well. Connect barbitol buffer solution (electrophoresis buffer solution) to the plate by using three layers of filter paper. Perform electrophoresis at a constant voltage of 100 V for about 2 hours (until the indicator is migrated to the front edge). Dig a trough at a width of 3 mm between the two wells. Each end of the trough is 3-5 mm apart from the edge of the plate. Add antiserum into the trough

until the trough is full. Put the plate in a wet box for diffusion at 37°C for 24 hours. After diffusion immerse the plate into physiological saline to remove the unbound protein. Stain the plate with 0.5% amino black staining solution and decolourize with the destaining solution until the background is basically colourless. Preserve by a suitable method or copy the profile. Compared with that of control, the main precipitation line of test sample shall be the protein to be tested.

Precaution

(1) Cooling system shall be used during electrophoresis, otherwise the agarose may be cracked due to drying.

(2) The plate shall be immersed sufficiently with physiological saline, otherwise the background is unclear.

VIII E Peptide Mapping

The integrity and accuracy of protein primary structure is characterized by suitable analytical methods after the protein has been cleaved by protease or chemical substance.

Method 1 Trypsin Cleavage-Reverse Phase HPLC Method

High performance liquid chromatography (Appendix III B) is applied for the characterization.

Parameters for chromatography

The column filled with octylsilane or octadecyl silane groups chemically bonded to porous silica particles for protein or peptide analysis is used. The column temperature is 30°C \pm 5°C and the test sample is preserved at 4°C \pm 0.5°C. The solution A of mobile phase is 0.1% trifluoroacetic acid in water and solution B of mobile phase is 0.1% trifluoroacetic acid in acetonitrile. Elute with a gradient (solution A from 100% to 30% and solution B from 0% to 70%) for 70 minutes at a flow rate of 1 ml per minute and detect at 214 nm.

Procedure

Dialyze the test sample and reference solutions, each at a concentration of 1 mg per ml, sufficiently against 1% ammonium bicarbonate solution. If the concentration of the test sample and reference substance is too low, they shall be concentrated separately to the corresponding concentration. Add trypsin solution (Dissolve a quantity of trypsin, pretreated with L-1-4'-tosylamino-2-phenylethyl chloromethyl ketone or of pure grade for sequencing, in 1% ammonium bicarbonate solution to prepare a solution containing 0.1 mg per ml) to test sample solution and reference solution separately at a ratio of 1 : 50 (mg/mg) and incubate at 37°C for 24 hours. Add 50% acetic acid solution at a ratio of 1 : 10. Centrifuge at 10000 r/min for 5 minutes (or filter with a 0.45 μ m membrane)

and collect the supernatant. Measure accurately 100 μ l of the supernatant (or filtrate) and inject into the HPLC apparatus separately. Elute with a gradient and record the chromatogram. Compare the chromatograms of test sample solution with those of reference solution.

Method 2 Cyanogen Bromide Cleavage Method

Procedure

Dialyze a quantity of test sample and reference solutions, equivalent to 50 μ g of protein, against water for 16 hours and lyophilize. Dissolve with 20 μ l of cyanogen bromide cleavage solution [Dissolve 0.3 g of cyanogens bromide in 1 ml of formic acid (70 \rightarrow 100)], allow to stand at room temperature for 24 hours. Add 180 μ l of water to the lysate and lyophilize again. Reconstitute the freeze-dried lysate with water to an appropriate concentration and perform SDS-PAGE (Appendix IV C) (Gel concentration is 20%). Silver staining is applied.

Compare the chromatograms of test sample with those of reference.

VIII F Determination of F(ab)₂ Content in Antitoxin

The content of components in test sample is determined quantitatively using thin layer scanning according to the principle of electrophoresis.

Reagent

- (1) Acrylamide solution; Dissolve 14 g of acrylamide and 0.367 g of bis-acrylamide in water to make 50 ml.
- (2) Phosphate buffer solution; Dissolve 17.8 g of sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), 103 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 4 g of sodium dodecyl sulfate in water to make 2000 ml.
- (3) 10% sodium dodecyl sulfate (SDS) solution
- (4) Sample binding solution; Dilute 4 ml of 10% SDS, 0.2 ml of phosphate buffer solution and 10 ml of glycerol to 20 ml with water.
- (5) Electrode buffer solution; Dilute the above-mentioned phosphate buffer solution doubly with water.
- (6) Fixing solution; Mix 400 ml of methanol with 70 ml of glacial acetic acid and dilute to 1000 ml with water.
- (7) Staining solution; Dissolve 2.5 g of Coomassie brilliant blue in 500 ml of methanol. Add 70 ml of glacial acetic acid and dilute to 1000 ml with water.
- (8) Destaining solution; Mix 70 ml of glacial acetic acid with 50 ml of methanol and dilute to 1000 ml with water.
- (9) Drying solution; Mix 30 ml of glycerol with 200 ml of methanol and dilute to 1000 ml with water.

Preparation of test sample solution

To 0.1 ml of the test sample at a certain protein concentration, add 0.1 ml of sample binding solution. Heat in 100°C water bath for 2 minutes or in 37°C water bath for 2 hours and then cool down.

Procedure

Add one to two drops of N, N, N', N'-tetramethylethyl-enediamine (TEMED) to the gel solution [prepared by mixing phosphate buffer solution, acrylamide solution, water and 1.5% ammonium persulfate solution at a volume ratio of 2 : 1 : 0.8 : 0.25]. Mix well and add to the slit between the two glass plates of electrophoresis trough. Care shall be taken to avoid the formation of air bubbles. Add a treated test sample containing 25 μ g of protein to each test sample well and then perform electrophoresis. A constant current of 2-3 mA shall be applied to each well. Electrophoresis shall be stopped when bromophenol blue reaches to the bottom.

After electrophoresis, put the gel plate in fixing solution overnight. Stain the gel in the staining solution for 1-2 hours and decolourize in the destaining solution until the background is colourless. Scan the gel plate at 575 nm by using a scanner to obtain (or calculate) the F(ab)₂ content of the test sample.

VIII G Determination of Molecular Size for Group A Meningococcal Polysaccharide

Method 1 Phosphorus Determination Method (arbitrary method)

The method is used for the determination of distribution coefficient (K_D) of bacterial capsular polysaccharide on chromatographic column and the recovery of polysaccharide before the specified K_D value is reached.

Reagent

- (1) Mobile phase
Dissolve 11.7 g of sodium chloride and 0.1 g of sodium azide in water and dilute to 1000 ml. Adjust pH to 7.0 with 0.1 mol/L sodium hydroxide solution.
- (2) Blue dextran 2000 solution
Dissolve 20 mg of blue dextran 2000 in the mobile phase solution and dilute to 10 ml.
- (3) Vitamin B₁₂ solution
Dissolve 10 mg of vitamin B₁₂ in the mobile phase solution and dilute to 10 ml.

Preparation of chromatographic column

Add 400 ml of the mobile phase to about 200 ml of agarose 4B or CL-4B gel and stir sufficiently. Precipitate by standing for about one hour. Discard the particles suspended in the upper layer.

Repeat the above procedure for 3-5 times. Add 200 ml of the mobile phase and mix well. Degas and pack the chromatographic column (1.5 cm × 90 cm) to a height of about 87 cm. To equilibrate the column, elute with the mobile phase solution at a rate of 15-20 ml per hour and about 500 ml of eluent (double to triple bed volume) is used.

Calibration of chromatographic column

Load 1 ml of blue dextran 2000 solution into the equilibrated column and elute with the mobile phase at a rate of 15-20 ml per hour. Collect the eluent with a fraction collector, 3 ml for each tube. Read the absorbance of each tube at 260 nm by ultraviolet-visible spectrophotometry (Appendix II A). Draw a graph using eluent volume (ml) as abscissa and absorbance as ordinate. The eluent volume of the peak at 260 nm is void volume V_o . Carry out the same procedure with 1 ml of vitamin B₁₂ solution starting from loading into the equilibrated column. The eluent volume of the peak at 370 nm is bed volume V_i .

Procedure

Load about 1 ml of test sample (containing 3-5 mg of polysaccharide antigen. If the sample is freeze-dried, it shall be reconstituted with the mobile phase) into the calibrated chromatographic column and elute with the mobile phase. Collect the eluent with a fraction collector, 5 ml for each tube. Determine the phosphorus content (Appendix VII A) of each tube. Plot a graph using eluent volume (ml) of each tube as abscissa and phosphorus content as ordinate. The eluent volume of the main peak is V_e .

Calculate the distribution coefficient K_D of test sample according to the following equation:

$$\text{Distribution coefficient of test sample} = \frac{(V_e - V_o)}{(V_i - V_o)}$$

Where: V_e = Eluent volume of test sample, ml;
 V_o = Void volume, ml;
 V_i = Bed volume, ml

Calculate the recovery of polysaccharide for test sample while K_D is less than 0.5 by the following equation:

$$R_x (\%) = A_x \times 100\% / A_t$$

Where: R_x = Recovery of polysaccharide of test sample while K_D is less than 0.5;
 A_x = Sum of phosphorus content of eluent in tubes while K_D is less than 0.5;
 A_t = Sum of phosphorus content of eluent in all tubes.

Method 2 Instrument Method

Preparation of reagent and chromatographic column
 The method and procedures are the same as those described in Method 1.

Calibration of chromatographic column

Mix 1 ml of blue dextran 2000 solution with 0.2 ml of vitamin B₁₂ and load into the equilibrated column. Elute with the mobile phase at a flow rate

of 15-20 ml per hour and detect at 206 nm. Collect the eluent with a fraction collector and record the chromatogram. In the chromatogram, the first peak is the peak of blue dextran 2000 and the volume of eluent at the peak is the void volume V_o . The second peak is the peak of vitamin B₁₂ and the volume of eluent at the peak is bed volume V_i .

Procedure

Load about 1 ml of test sample (containing 3-5 mg of polysaccharide antigen. If the sample is freeze-dried, it shall be reconstituted with the mobile phase) into the calibrated chromatographic column. Elute with the mobile phase at a flow rate of 15-20 ml per hour and detect at 206 nm. Collect the eluent with a fraction collector, 5 ml for each tube, and record the chromatogram.

Calculate the distribution coefficient K_D of test sample by the following equation:

$$\text{Distribution coefficient of test sample} = \frac{(V_e - V_o)}{(V_i - V_o)}$$

Where: V_e = Eluent volume of test sample, ml;
 V_o = Void volume, ml;
 V_i = Bed volume, ml

Calculate the recovery of polysaccharide of test sample while K_D is less than 0.5 by the following equation:

$$R_x (\%) = A_x / A_t \times 100\%$$

Where: R_x = Recovery of polysaccharide of test sample while K_D is less than 0.5;
 A_x = Chromatogram areas of test sample while the K_D is less than 0.5;
 A_t = Sum of total area of chromatogram for test sample.

[Note] The chromatography shall be carried out at 10-20°C.

VIII H Determination of Molecular Size for Typhoid Vi Polysaccharide

The method is used for the determination of distribution coefficient (K_D) of bacterial capsular polysaccharide in chromatographic column and the recovery of polysaccharide before the specified K_D value is reached.

Reagents and preparation and calibration of chromatographic column

See Method 2 in Appendix VIII G.

Procedure

Load about 1 ml of test sample (containing 3-5 mg of polysaccharide antigen) into the calibrated chromatographic column and elute with the mobile phase at a flow rate of 15-20 ml per hour. Collect the eluent with a fraction collector, 3-5 ml per tube. Determine the O-acetyl content in each tube according to the test for determination of O-acetyl content (Appendix VI F). The eluent volume

with the maximum O-acetyl content is served as the eluent volume of polysaccharide main peak (V_e).

The distribution coefficient K_D of test sample is calculated by the following equation;

$$K_D = (V_e - V_o) / (V_i - V_o)$$

Where: K_D = Distribution coefficient of test sample;

V_e = Eluent volume for test sample, ml;

V_o = Void volume, ml;

V_i = Bed volume, ml

Calculate the recovery of polysaccharide of test sample while K_D is not more than 0.25 by the

following equation;

$$R_x (\%) = A_x / A_t \times 100\%$$

Where: R_x = Recovery of polysaccharide of test sample when the K_D is not more than 0.25

A_x = O-acetyl content of eluent pooled in equal volumes from each tube when the K_D of test sample is not more than 0.25

A_t = O-acetyl content of eluent pooled in equal volumes from each tube

[Note] The chromatography shall be carried out at 10-20°C.

Appendix IX

IX A Determination of Residual Antibiotics

Antibiotic may inhibit the growth of microbe on agar culture medium. The residual ampicillin or tetracycline content in test sample is determined by comparing the size of bacteriastatic rings formed by test sample and standard against corresponding bacteria which grow on culture medium.

Preparation of phosphate buffer solution (pH 6.0)

Dissolve 8.0 g of potassium dihydrogen phosphate and 2.0 g of dipotassium hydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 30 minutes.

Preparation of antibiotic No. 2 medium

Dissolve 6 g of peptone, 1.5 g of beef extract powder and 6 g of yeast extract powder in a quantity of water. Add 13 - 14 g of agar and swell by heating. Filter to remove the insoluble matters. Dissolve 1 g of glucose in the mixture and dilute to 1000 ml with water. Adjust pH so that after sterilization it is 6.5 - 6.6. Dispense the medium in glass tubes or conical flasks. Sterilize at 115°C for 30 minutes and store at 4°C.

Preparation of standard solution

Dissolve a quantity of ampicillin standard with 0.01 mol/L hydrochloric acid and dilute to a concentration of 10 mg of ampicillin per ml. Measure accurately a quantity of the above ampicillin solution and dilute with phosphate buffer solution to a concentration of 1.0 µg of ampicillin per ml.

Dissolve a quantity of tetracycline standard with physiological saline and dilute to a concentration of 0.125 µg of tetracycline per ml.

Preparation of bacterial suspension

(1) *Staphylococcus aureus* suspension It is used for the determination of ampicillin.

Inoculate the nutrient agar slant culture of *Staphylococcus aureus* (CMCC 26003) strain onto nutrient agar slant and incubate at 35 - 37°C for 20 - 22 hours. Immediately before use, wash off the bacterial lawn with sterilized water or sterile 0.9% sodium chloride solution.

(2) *Micrococcus luteus* suspension It is used for

the determination of tetracycline.

Inoculate the nutrient agar slant culture of *Micrococcus luteus* (CMCC 28001) strain onto nutrient agar slant and incubate at 26-27°C for 24 hours. Immediately before use, wash off the bacterial lawn with sterile 0.9% sodium chloride solution.

Procedure

Pour 15 - 20 ml of melted antibiotic medium No. 2 into a Petri dish 8 cm or 10 cm in diameter and spread evenly on the dish bottom. Put the dish on a horizontal platform and let the medium solidify to form a bottom layer. Transfer 10-15 ml of antibiotic medium No. 2 to a test tube preheated in 50°C water bath. Add 300 µl of 0.5%-1.5% (ml/ml) of bacteria suspension and mix well. Pour a quantity of the mixture onto the Petric dish spread with a bottom layer. Put the dish on a horizontal platform and cool down. Place steel tubes (stainless steel tubes of smooth surface, with an inner diameter of 6 - 8 mm, a height of 10-15 mm and a wall thickness of 1-2 mm) evenly and apart with equal distances on each Petric dish. Add test sample solution, negative control solution (phosphate buffer solution) and standard solution dropwise into the steel tubes separately. Incubate the Petric dishes at 37°C for 18 - 22 hours.

Result evaluation

There is bacteriastatic ring for standard solution but not for negative control solution. The result is judged as negative if the diameter of bacteriastatic ring of test sample solution is smaller than that of standard solution, otherwise it is judged as positive.

[Note]

The test shall be carried out aseptically. All the glass apparatus and steel tubes used for the test shall be sterile.

IX B Determination of Residual Extraneous DNA

The residual extraneous DNA in test sample is denatured to a single stranded and adsorbed onto an immobilized membrane. Under a certain temperature, the single strand binds with a matched single strand and reassociates to form a double

stranded DNA, which is called as hybridization. A labelled specific single stranded DNA probe is hybridized with the single stranded DNA of test sample, which is adsorbed onto an immobilized membrane. The hybridization result is shown by a display system corresponding to the label. The DNA content of test sample can be determined by comparing the result with that of positive DNA reference substance of known DNA content.

Reagent

(1) DNA labeling and detection kit.

(2) DNA hybridization membrane

Nylon membrane or nitrocellulose membrane.

(3) 2% Proteinase K solution

Dissolve 0.20 g of proteinase K in 10 ml of sterile water. Dispense the solution and store at -20°C for use.

(4) 3% Bovine serum albumin solution

Dissolve 0.30 g of bovine serum albumin in 10 ml of sterile water.

(5) 1 mol/L Tris (hydroxymethyl) amino-methane (Tris) solution (pH 8.0)

Adjust pH to 8.0 with hydrochloric acid.

(6) 5.0 mol/L sodium chloride solution

(7) 0.5 mol/L disodium ethylenediamine tetraacetate solution (pH 8.0)

Adjust pH to 8.0 with 10 mol/L sodium hydroxide solution.

(8) 20% sodium dodecyl sulfate (SDS) solution

Adjust pH to 7.2 with hydrochloric acid.

(9) Proteinase buffer solution (pH 8.0)

Mix 1.0 ml of 1 mol/L Tris solution (pH 8.0), 2.0 ml of 5.0 mol/L sodium chloride solution, 2.0 ml of 0.5 mol/L disodium ethylenediamine tetraacetate solution (pH 8.0) with 2.5 ml of 20% SDS solution and dilute to 10 ml with sterile water.

(10) TE buffer solution (pH 8.0)

Dilute 10 ml of 1 mol/L Tris solution (pH 8.0) and 2 ml of 0.5 mol/L disodium ethylenediamine tetraacetate solution (pH 8.0) to 1000 ml with sterile water.

(11) 1% Protamine DNA solution

Weight accurately 0.10 g of protamine DNA and dissolve in a small volume of TE buffer solution. Transfer totally to a 10 ml volumetric flask and dilute to volume with TE buffer solution. Mix well. Shear the DNA into small molecules by drawing and blowing repeatedly using a syringe fitted with a No. 7 needle. Dispense the solution and store at -20°C .

(12) DNA dilution solution

Dilute 50 μl of 1% protamine DNA solution to 10 ml with TE buffer solution.

Preparation of DNA

DNA used for probe labeling and used as DNA reference substance is obtained by extraction and purification from continuous cell line, engineering bacteria or hybridoma cells where the test sample is produced. The methods of purification and identification may refer to *Molecular Cloning*, A

Laboratory Manual, 2002, or *Short Protocols in Molecular Biology*, 1998.

Adjust the cell suspension for extraction to a concentration of 10^7 cells per ml. If the host cell is bacteria, the concentration shall be adjusted to 10^8 bacteria per ml. Centrifuge 1 ml of the suspension. Add 400 μl of lysis solution to the precipitate and mix well. React at 37°C for 12-24 hours. Add 450 μl of saturated phenol solution and mix vigorously. Centrifuge at 10000 r/min for 10 minutes. Discard the supernatant and extract with 450 μl of saturated phenol solution once more. Discard the supernatant, add 450 μl of trichloromethane and mix vigorously. Centrifuge at 10000 r/min for 10 minutes. Discard the supernatant, add 40 μl of 3 mol/L sodium acetate (pH 5.2) and mix well. Then, add 1 ml of cold absolute ethanol at a temperature lower than -20°C and mix sufficiently. React for 2 hours at a temperature lower than -20°C . Centrifuge at 15000 r/min for 15 minutes and wash the precipitate once with a quantity of 70% cold ethanol at a temperature lower than -20°C . Centrifuge at 15000 r/min for 15 minutes and discard the supernatant. Dry the precipitate by air blowing and dissolve with a quantity of sterile TE buffer solution. Cleave the RNA with RNase, extract with phenol/trichloromethane and purify with molecular sieve chromatography.

Identify the DNA purity of the reference substance by electrophoresis on 1% agarose gel and spectrophotometry; RNA or oligonucleotide shall not be detected; the ratio of A_{260} to A_{280} shall be in the range of 1.8 - 2.0 (The test sample is diluted to A_{260} between 0.2 and 1.0 for testing).

The DNA used for the reference substance and probe labeling shall be cleaved with enzyme or treated by ultrasonication in order to make the size of its fragments suitable for DNA hybridization and probe labeling.

The DNA concentration of the reference substance is calculated by the following formula:

$$\text{DNA concentration (ng/}\mu\text{l)} = 50 \times A_{260}$$

The reference substance may be dispensed into suitable tubes. Store at -20°C for long-term use. Probe labeling: Carry out according to the instructions of the reagent kit.

Procedure

(1) Proteinase K pre-treatment

Prepare test sample, reference substance and negative control according to the following table. Warm at 37°C for more than 4 hours to ensure that the reaction of enzyme cleavage is completed.

Tube	Volume of test sample, μl	2% Proteinase K, μl	Proteinase buffer solution, μl	3% BSA solution, μl	Water, add to, μl
Sample	100	1	20		200
D ₁	100	1	20	X	200
D ₂	100	1	20	X	200

Continued

Tube	Volume of test sample, μl	2% Proteinase K, μl	Proteinase buffer solution, μl	3% BSA solution, μl	Water, add to, μl
D ₃	100	1	20	X	200
Negative	100	1	20	X	200

Precaution

The dilution of test sample: Dilute the test sample (bulk preparation) with DNA dilution solution to a concentration of one human dose per 100 μl ; if the maximum dosage of final product is comparatively large and the protein concentration of the test sample is comparatively low, the test sample may be diluted with DNA dilution to a concentration of 1/10 or 1/100 human dose per 100 μl .

D₁, D₂, and D₃ are diluted DNA reference substances. The DNA reference substance is diluted with DNA dilution solution to a concentration of 1000 ng of DNA per ml and then three successive 10-fold dilutions are made to obtain the concentrations of 10 ng/100 μl (D₁), 1 ng/100 μl (D₂) and 100 pg/100 μl (D₃); if the dosage of final product is comparatively large and the DNA content limit (100 pg/dose) is strict, the sensitivity of DNA test shall be elevated. Then the corresponding DNA reference substance shall be diluted to 100 pg/100 μl (D₁), 10 pg/100 μl (D₂) and 1 pg/100 μl (D₃). The negative control is DNA dilution solution and the blank control is TE buffer solution, which has not been pretreated with proteinase K.

When 1/100 human dose of test sample is greater than 100 μl , the final volume also increases. Usually, the final volume is around twice the volume of test sample. If the volume difference between the test sample and final volume is too small, the activity of proteinase K may be influenced.

The volume ratio of 2% proteinase K solution to proteinase buffer solution is 1/20 and that of proteinase buffer solution to final volume is 1/10. Add a quantity of 3% bovine serum albumin so that the reference substance and negative control may contain a certain amount of protein and maintain the same enzyme cleavage conditions as those for the test sample (usually protein in nature). If other substances are used as the test samples, corresponding substances shall be used. If protein in the pretreated test sample solution interferes with the test, the DNA in test sample may be extracted by the above saturated phenol solution extraction method or by other suitable methods (extraction shall also be carried out again for the DNA of reference substance and negative control in parallel with the test sample).

(2) Dot blotting or slot blotting

After the hybridization membrane is immersed in TE solution, heat the pretreated test sample, reference substance and negative control and blank

control in 100°C water bath for 10 minutes. Cool down in an ice bath promptly. Centrifuge at 8000 r/min for 5 seconds. Load the test sample and all controls onto the hybridization membrane by dot blotting or slot blotting unit (as protein precipitate exists, the loading quantity is based on the amount of precipitate and the loading of protein precipitates should be avoided; the volume for the test sample, reference substance, negative control and blank control shall be identical or in the same proportion).

Air-dry the membrane and then heat at 80°C under vacuum for more than one hour.

(3) Hybridization and colour development

Perform according to the instructions of the reagent kit.

Result evaluation

The test is valid if the reference substances develop colour and the intensity of colour corresponds with the DNA content, and if the negative control and blank control do not develop colour or the colour developed is lighter than that of the reference substance (D₃). Compare the test sample with reference substances and evaluate the residual extraneous DNA content of test sample according to the colour intensity.

IX C Determination of Residual Host Bacterial Protein (*E. coli*)

The content of residual host bacterial protein in recombinant products prepared by *E. coli* expression system is determined by ELISA.

Reagent

(1) Coating solution (carbonate buffer solution, pH 9.6)

Dissolve 0.32 g of sodium carbonate and 0.586 g of sodium bicarbonate in water and dilute to 200 ml.

(2) Phosphate buffer solution (pH 7.4)

Dissolve 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 500 ml. Sterilize at 121°C for 15 minutes.

(3) Washing solution (pH 7.4)

Dilute 0.5 ml of polysorbate 20 to 500 ml with phosphate buffer solution.

(4) Dilution solution (pH 7.4)

Dissolve 0.5 g of bovine serum albumin in washing solution and dilute to 100 ml.

(5) Concentrated dilution solution

Dissolve 1.0 g of bovine serum albumin in washing solution and dilute to 100 ml.

(6) Substrate buffer solution (citric acid-phosphate buffer solution, pH 5.0)

Dissolve 1.84 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 0.51 g of citric acid in

water and dilute to 100 ml.

(7) Substrate solution

Dissolve 8 mg of o-phenylenediamine in a quantity of substrate buffer solution. Add 30 μ l of 30% hydrogen peroxide and dilute to 20 ml with substrate buffer solution. Prepare just before use.

(8) Stopping solution

1 mol/L sulfuric acid.

Preparation of standard solution

Reconstitute the *E. coli* host cell protein standard with water according to the instructions and dilute with dilution solution to 500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 15.625 ng and 7.8125 ng of host cell protein per ml separately.

Preparation of test sample

Dilute a quantity of test sample to a concentration of about 250 μ g per ml. If the concentration of the test sample is lower than 500 μ g per ml, dilute with an equal volume of concentrated dilution solution.

Procedure

Dilute a volume of rabbit anti-*E. coli* host cell protein antibody to 10 μ g per ml with coating solution. Add 100 μ l of the diluted antibody to each well of a 96-well microtiter plate. Allow to stand overnight at 4°C (16 - 18 hours). Wash the microtiter plate 3 times with washing solution. Prepare a 1% bovine serum albumin solution with washing solution and add 200 μ l of the solution to each well of the 96-well microtiter plate. Incubate at 37°C for 2 hours. Wash the blocked microtiter plate 3 times with washing solution. Add 100 μ l of the standard and test sample solutions into each well of the plate separately, in duplicate. Meanwhile, add dilution solution into other two wells as a blank control. Incubate at 37°C for 2 hours. Dilute rabbit anti-*E. coli* host cell antibody, labelled with horseradish peroxidase (HRP), with dilution solution at a ratio of 1 : 1000. Add 100 μ l of the diluted solution into each well of the plate. Allow to stand at 37°C for 1 hour. Wash the microtiter plate 10 times with washing solution. Add 100 μ l of substrate solution into each well of the plate. Allow to stand at 37°C for 40 minutes, protected from light. Add 50 μ l of stopping solution into each well of the plate to stop the reaction. Read the absorbance of each well at 492 nm and analyze the data with a computer software or calculate the result manually with graphic method.

Plot a curve with concentration of standard solution against the corresponding absorbance. Find out the host cell protein content on the curve through its absorbance. Calculate the residual host cell protein content in test sample by the following equation.

Residual host cell protein content of test sample (%) = $C \times D \times 100\% / T \times 10^6$

Where: C = Residual host cell protein content of test sample solution, ng/ml;

D = Dilution factor of test sample;

T = Protein content of test sample, mg/ml.

IX D Determination of Residual Host Bacterial Protein (*Pseudomonas*)

The content of residual host bacterial protein in recombinant products prepared by *Pseudomonas* expression system is determined by ELISA.

Reagent

(1) Coating solution (carbonate buffer solution, pH9.6): Weigh accurately 0.32 g of sodium carbonate and 0.586 g of sodium hydrogen carbonate, transfer into a 200 ml volumetric flask, dissolve with water and dilute to the volume.

(2) Phosphate buffer saline: Transfer 8.0 g of sodium chloride, 0.20 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate into a 500 ml volumetric flask, dissolve with water and dilute to the volume. Sterilize at 121°C for 15 minutes.

(3) Washing solution: Dilute 0.5 ml of polysorbate 20 to 500 ml with phosphate buffer saline.

(4) Concentrated diluent: Dissolve 1.0 g of bovine serum albumin in washing solution and dilute to 100 ml.

(5) Diluent: Dilute the concentrated diluent with an equal volume of water.

(6) Substrate buffer solution (0.005 mol/L sodium acetate-citric acid buffer solution): Dissolve 0.68 g of sodium acetate and 1.05 g of citric acid ($C_6H_8O_7 \cdot H_2O$) in water and dilute to 1000 ml. Adjust pH to 3.6.

(7) Substrate solution A: Dissolve 0.08 g of 3,3',5,5'-tetramethylbenzidine (TMB) in 40 ml of dimethyl sulfoxide (DMSO), add 60 ml of methanol and mix well. Add 100 ml of substrate buffer solution, stir in a dark place for 2 hours until the mixture is dissolved completely. Allow the solution to stand at room temperature for 4 hours.

(8) Substrate solution B: Dilute 3.2 ml of 1.5% hydrogen peroxide to 1000 ml with substrate buffer solution.

(9) Substrate solution: Mix substrate solutions A and B at equal volumes just before use.

(10) Stopping solution: 2 mol/L sulfuric acid solution.

Preparation of reference solution

Dissolve host bacterial protein reference with the diluent following the instructions of reagent kit, then measure accurately a quantity of the solution and dilute with diluent to host bacterial protein concentrations of 20 ng, 10 ng, 5 ng, 2.5 ng, 1.2 ng, 0.6 ng and 0.3 ng per ml.

Preparation of test sample solution

Dilute a quantity of test sample with the diluent to

a concentration of about 100 μg of protein per ml. If the protein concentration is less than 200 $\mu\text{g}/\text{ml}$, the test sample shall be diluted with an equal volume of concentrated diluent.

Procedure

Dilute the coating antibody to a suitable concentration with the coating solution. The dilution factor is indicated in the instructions of the reagent kit. Add 100 μl of the diluted antibody into each well of a 96-well microtitre plate, allow the plate to stand at 2-8°C for 16-20 hours. Wash the microtitre plate 3 times with washing solution. Prepare a 1% bovine serum albumin (BSA) solution with washing solution. Add 200 μl of BSA solution into each well of the plate and oscillate (200-300 r/min) at room temperature for one hour. Wash the plate 3 times with washing solution. Add the reference and test sample solutions into the wells of the plate separately, 100 μl per well in duplicate. Meanwhile, add diluent into other two wells as blank controls. Oscillate (200-300 r/min) the microtitre plate at room temperature for one hour, and wash 3 times with washing solution. Dilute the primary antibody with diluent to a suitable concentration following the instructions of reagent kit. Add 100 μl of the diluted antibody into each well of the microtitre plate and oscillate (200-300 r/min) at room temperature for one hour. Wash the microtitre plate 3 times with washing solution. Dilute horseradish peroxidase (HRP)-labelled secondary antibody with diluent to a suitable concentration following the instructions of reagent kit. Add 100 μl of the diluted antibody into each well of the microtitre plate and oscillate (200-300 r/min) at room temperature for 30 minutes. Wash the microtitre plate 8 times with washing solution. Add 100 μl of substrate solution into each well and set the microtitre plate at room temperature to react for 10-15 minutes, protect from light. Add 100 μl of stopping solution into each well to stop the reaction. Read the absorbance of each well at 450 nm with a microtitre plate reader and analyze the data with a computer software or calculate the result manually with graphic method.

Plot a curve with the concentrations of the reference solutions against their absorbance. Find out the host bacterial protein content of test sample solution on the curve through its absorbance. Calculate the residual host bacterial protein content in the test sample by the following formula.

Residual host bacterial protein content (%) in the test sample = $(C \times D) / (T \times 10^6) \times 100\%$

Where: C = Host bacterial protein concentration in the test sample, ng/ml;

D = Dilution factor of the test sample;

T = Total protein content in the test sample, mg/ml.

IX E Determination of Residual Host Yeast Protein

The content of residual host yeast protein in recombinant product prepared by yeast expression system is determined by ELISA.

Reagent

(1) Coating solution (carbonate buffer solution, pH9.6): Dissolve 0.32 g of sodium carbonate and 0.586 g of sodium bicarbonate in water and dilute to 200 ml.

(2) PBS: Dissolve 8.0 g of sodium chloride, 0.20 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Adjust pH to 7.4 and sterilize at 121°C for 15 minutes.

(3) Washing solution (PBS-Tween 20): Dilute 0.5 ml of polysorbate 20 to 1000 ml with PBS.

(4) Diluent: Dissolve 0.5 g of bovine serum albumin with washing solution and dilute to 100 ml.

(5) Substrate buffer solution (0.005 mol/L sodium acetate-citric acid buffer solution): Dissolve 0.68 g of sodium acetate and 1.05 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) in water and dilute to 1000 ml. Adjust pH to 3.6.

(6) Substrate solution A: Dissolve 0.08 g of 3, 3', 5, 5'-tetramethylbenzidine (TMB) in 40 ml of dimethyl sulfoxide (DMSO), add 60 ml of methanol and mix well. Add 100 ml of substrate buffer solution and stir in a dark place for 2 hours until the mixture is dissolved completely. Allow the solution to stand at room temperature for 4 hours.

(7) Substrate solution B: Dilute 3.2 ml of 1.5% hydrogen peroxide to 1000 ml with substrate buffer solution.

(8) Substrate solution: Mix substrate solutions A and B at equal volumes just before use.

(9) Stopping solution: 1 mol/L sulfuric acid solution.

Preparation of reference solution

Reconstitute the reference with water, then measure accurately a quantity of the solution and dilute with diluent to make 1 ml containing 1000 ng, 500 ng, 250 ng, 125 ng and 62.5 ng of host yeast protein.

Preparation of test sample solution

Dilute a quantity of test sample with the diluent to a suitable concentration.

Procedure

Dilute a quantity of guinea pig anti-yeast protein antibody with coating solution to a suitable concentration. Add 100 μl of the diluted antibody into each well of a 96-well microtitre plate. Seal

the plate with a piece of film, allow to stand at 4°C overnight. Wash the plate 3 times with washing solution. Prepare a 1% bovine serum albumin (BSA) solution with washing solution. Add 200 µl of 1% BSA solution into each well of the plate, allow to stand at 37°C for 2 hours. Wash the blocked plate 3 times with washing solution. Add the reference and test sample solutions into the wells of the microtitre plate separately, 100 µl per well in duplicate. Meanwhile, add diluent into other two wells as blank controls. Seal the microtitre plate with a piece of film, allow to stand at 37°C for one hour. Wash the plate 6 times with washing solution. Dilute rabbit anti-yeast antibody with diluent to a suitable concentration. Add 100 µl of the diluted antibody into each well of the microtitre plate. Seal the microtitre plate with a piece of film, allow to stand at 37°C for one hour. Wash the plate 6 times with washing solution. Dilute horseradish peroxidase (HRP) -labelled sheep anti-rabbit antibody (IgG-HRP) solution with the diluent to a suitable concentration. Add 100 µl of the diluted IgG-HRP solution into each well. Seal the microtitre plate with a piece of film, allow to stand at 37°C for one hour. Wash the plate 6 times with washing solution. Add 100 µl of substrate solution into each well, allow to stand at room temperature for 5-10 minutes, protected from light. Add 100 µl of stopping solution into each well to stop the reaction. Read the absorbance of each well at 450 nm with a microtitre plate reader, using 630 nm as a reference wavelength. Analyze the data with a computer software or calculate the result manually with graphic method. Plot a curve with the concentrations of the reference solutions against their absorbance. Find out the host yeast protein content of sample solution on the curve through its absorbance. Calculate the residual host yeast protein content of the test sample by the following formula.

$$\text{Residual host yeast protein content (\%)} = \frac{(C \times D)}{(T \times 10^6)} \times 100\%$$

Where: C = Host yeast protein concentration of test sample, ng/ml;

D = Dilution factor of test sample;

T = Total protein content of test sample, mg/ml.

IX F Determination of Prekallikrein Activator Content

The content of prekallikrein activator (PKA) in test sample is determined by chromogenic substrate method (or chromogenic matrix method).

Reagent

(1) 0.05 mol/L tris(hydroxymethyl)aminomethane-

0.15 mol/L sodium chloride solution (TNB); Adjust pH to 8.0 with 1 mol/L hydrochloric acid solution.

(2) 2 mmol/L kallikrein chromogenic substrate (S-2302) solution: Dissolve 12.5 mg of S-2302 in 10 ml of water.

(3) Prekallikrein (PK): Prepare PK by a suitable method, dispense it in small volumes and store at -30°C or below for use.

Preparation of PKA standard solution

Dilute a quantity of PKA standard with 0.85% sodium chloride solution to contain 10.0 IU, 20.0 IU, 30.0 IU, 40.0 IU and 50.0 IU per ml respectively. According to the required amount for each test, dispense the standard solution in small volumes and store at -30°C. Thaw before use and dilute 10-fold with TNB. Each tube can only be thawed once.

Preparation of test sample solution

Dilute the test sample 10-fold with TNB.

Procedure

To a 96-well microtitre plate, add 20 µl of test sample solution and 20 µl of PK. Oscillate the microtitre plate for one minute and cover with a lid, allow to stand at 25-30°C for 30 minutes (or at 37°C for 10 minutes). To each reaction well, add 20 µl of 2 mmol/L S-2302 solution. Oscillate the plate for one minute and cover with a lid, allow to stand at 25-30°C for 15 minutes (or at 37°C for 10 minutes). Add 20 µl of 50% acetic acid solution to each well and oscillate for one minute. Read the absorbance of each well at 405 nm by ultraviolet-visible spectrophotometry (Appendix II A). At the same time, set up a blank control by using 20 µl of TNB instead of PK with the same procedure. Use 20 µl of PKA standard solution instead of test sample solution and repeat the procedure. A linear regression formula is obtained by regressing the logarithm of PKA activity of standard solutions with the logarithm of their corresponding absorbance. Calculate the PKA activity of test sample by the formula.

[Note]

(1) Three wells shall be set up for each standard and test sample, among them, two are the test wells, and the other one is the control well. The difference between absorbance of the two test wells shall be less than 0.020.

(2) According to the PKA content in the test sample, select a suitable dilution range of standard.

(3) The correlation coefficient of linear regression shall be not less than 0.99.

(4) Whenever PK, S-2302 and 50% acetic acid solutions are added to the wells, the time intervals of addition between wells, as well as the order of addition, shall be the same so as to allow each well react under the same conditions.

IX G Test for Losing Rate of Plasmid

The recombinant bacteria in *E. coli* expression system contain plasmids for the expression of target protein. In general, the plasmid carries antibiotic-resistant gene to facilitate selection. During subculture in the environment with a certain concentration of antibiotic, such as in seed culture medium, the recombinant bacterium can not survive after the plasmid is lost. However, in the fermentation medium without antibiotic, along with the increasing number of passage, a part of *E. coli* may lose the plasmid, as a result, the antibiotic-resistant gene and the ability to expressing target protein may lose at the same time. The stability of plasmid is evaluated by the losing rate of plasmid determined by comparing the numbers of survival bacteria in the media with or without antibiotic.

The real-time fermentation liquid during fermentation or mimic fermentation process, including those at the final stage of fermentation when the bacteria are subcultured to the maximum passage, are collected and used for the test in general. Dilute the fermentation liquid properly, spread to an antibiotic-free medium and incubate at 37°C overnight. Select at least 100 colonies on the medium and inoculate onto the Petric dishes with or without antibiotic respectively. Incubate at 37°C overnight and compare the numbers of colonies in the two Petric dishes and calculate the losing rate of plasmid. The test shall be repeated for more than 2 times in general. The losing rate of plasmid shall be specified in the validation of production procedure and shall be within the allowable range.

IX H Test for Nucleotide Sequence of SV40

The presence of SV40 nucleotide sequence in the test sample is detected by polymerase chain reaction (PCR) using two pairs of specific primers designed to amplify two fragments, i. e. 100 bp (2220-2319) of SV40 VP1 and 451 bp (2619-3070) at C-terminus of major T antigen.

Preparation of test sample and control solutions

Add 25 µl of 2% protease K solution, 50 µl of 10% SDS solution and 10 µl of 0.05 mol/L EDTA solution (pH8.0) into 400 µl of the test sample. Incubate at 56°C for one hour. Extract with an equal volume of mixture of phenol and trichloromethane (1:1), and extract again with an equal volume of trichloromethane. Then add two

volumes of ethanol. Keep at -20°C for 16 hours. Centrifuge at 10000 r/min for 15 minutes. Wash the precipitate with 75% ethanol and dry. Dissolve in 10 µl of DNase-free and RNase-free water. Positive control and negative control are treated by the above-mentioned method simultaneously with test sample.

Primers

Upstream primer for VP1: 2220 5'-ACA CAG CAA CCA CAG TGG TCC-3'2240

Downstream primer for VP1: 2319 5'-GTA AAC AGC CCA CAA ATG TCA AC-3'2297

Upstream primer for C-terminus of T antigen: 3070 5'-GAC CTG TGG CTG AGT TTG CTC A-3'3049

Downstream primer for C-terminus of T antigen: 2619 5'-GCT TTA TTT GTA ACC ATT ATA AG-3'2641

Procedure

(1) The amount of primer added to the test sample for amplification is 30×10^{-12} mol. 1 µl of DNA template is added and the final volume is 50 µl. PCR amplification parameters are as follows: Denature at 94°C for 3 minutes; a total of 40 cycles of 94°C for 20 seconds, 50°C for 20 seconds and 72°C for 40 seconds, with final extension of 72°C for 3 minutes.

(2) Detect the amplified products by electrophoresis with 2% agarose gel containing 1 µg of ethidium bromide per millilitre. Perform electrophoresis in 1×TAE buffer solution at 100 V for about 40 minutes. The length of the amplified fragment for VP1 is 100 bp, and that for C-terminus of major T antigen is 451 bp.

(3) Repeat the amplification of VP1 fragment with the same template. Care shall be taken to avoid nonspecific amplification due to contamination. Alternatively, transfer the amplified products and the controls from the gel to Hybond N nylon membrane for Southern blotting with VP1 probe in order to make sure that the amplified fragment is VP1.

(4) Sequence the C-terminus coding major T antigen of the amplified products in the test sample and in the positive control with automatic sequencer.

Result evaluation

The test is valid if the specific products are obtained in the positive control and no such products are found in the negative control. Criteria for evaluation are as follows.

The result is negative if no VP1 fragment is amplified.

If the VP1 fragment is amplified, the test shall be repeated. If no VP1 amplification is shown in the repeated test, the result is negative.

If VP1 amplification is positive in the repeat test, the fragment of C-terminus coding major T antigen shall be further amplified. If the fragment is amplified, its sequence shall be compared with that amplified from the positive control. If the two

sequences are identical, the result is judged as positive. If no fragment of C-terminus coding major T antigen is amplified, the test shall be repeated following the steps (1) to (3). If the assay fails again to amplify the C-terminus coding major T antigen, the result is judged as negative.

IX I Test for Blood Group A-like Substance (Hemagglutination Inhibition Assay)

The blood group A-like substance content in test sample is determined by reacting blood group A-like substance reference and test sample with anti-blood group A serum respectively and comparing the end-points of the two reactions.

1% blood group A erythrocyte suspension

Mix the blood of group A from six donors at equal volumes, add a quantity of physiological saline and mix well. Centrifuge at 2000 r/min for 10 minutes. Decant the supernatant and wash the precipitate 3 times with physiological saline. Mix 1 ml of the precipitate with 99 ml of physiological saline to make a 1% erythrocyte suspension.

Preparation of blood group A-like substance reference solution

The blood group A-like substance reference is distributed or accredited by the NCL. The reference solution is made to contain 1 mg per ml. In a group of ten test tubes 9 mm in diameter, carry out a series of 2-fold dilutions for 0.1 ml of blood group A-like substance reference with physiological saline starting from 1/100 dilution (0.01 mg/ml).

Preparation of test sample solution

In a group of ten test tubes 9 mm in diameter, carry out a series of 2-fold dilutions for 0.1 ml of test sample with physiological saline starting from the original concentration.

Determination of the testing dose of anti-blood group A serum

In a group of ten test tubes 9 mm in diameter, carry out a series of 2-fold dilutions for 0.1 ml of anti-blood group A serum with physiological saline starting from 1/2 dilution. To 0.1 ml of each dilution, add 0.1 ml of 1% blood group A human erythrocyte suspension. At the same time, prepare a negative control by adding 0.1 ml of blood group A human erythrocyte to 0.1 ml of physiological saline. Mix well by shaking, allow the mixtures to stand at room temperature for 15 minutes, centrifuge at 1500 r/min for one minute and observe the agglutination extent according to the status of cell sedimentation. The highest dilution of anti-blood group A serum which still shows complete agglutination (+ + + +) is served as one testing dose.

Procedure

To 0.1 ml of each dilution of test sample and blood group A-like substance reference, add 0.1 ml of anti-blood group A serum containing two testing doses and mix well by shaking. Allow the mixture to stand at 36.5-37.5°C for 10 minutes and then add 0.1 ml of 1% human blood group A erythrocytes into each tube. Mix well again by shaking, allow to stand at 36.5-37.5°C for 15 minutes. Centrifuge at 1500 r/min for one minute and observe the agglutination extent according to the sedimentation of erythrocytes.

Result evaluation

The content (mg) of blood group A-like substance in 1 ml of the sample is calculated by multiplying the highest dilution factor of test sample showing complete hemagglutination inhibition (end point) by the content of blood group A-like substance in the highest dilution of control sample showing the similar extent of hemagglutination inhibition.

IX J Test for Anti-A and Anti-B Hemagglutinins (Indirect Anti-human Globulin Test)

The anti-A and anti-B hemagglutinins in test sample are determined by indirect anti-human globulin test (Coombs test).

Reagent

(1) Erythrocyte suspension: Mix three samples of each group A, B and RhD-positive group O erythrocytes separately. Wash 3 times with physiological saline. After the last washing, centrifuge at 2000 r/min for 5 minutes. Measure a quantity of the deposit and prepare into a 5% (ml/ml) erythrocyte suspension with physiological saline. The erythrocyte shall be used within one week after blood collection.

(2) Anti-human globulin serum: Polyvalent anti-serum against human globulin shall be calibrated before use and diluted to an optimal dilution which is determined according to the instructions provided by the manufacturer or by the method described in the Note.

Procedure

Make a quantity of test sample a series of 2-fold dilutions. Set up two rows of small test tubes (75 mm×12 mm) for the test sample of each dilution. Add 0.2 ml of test sample to each tube. Add 0.2 ml of 5% group A erythrocyte suspension to each tube of the first row and 0.2 ml of 5% group B erythrocyte suspension to each tube of the second row, mix well, allow to stand in 37°C water bath for 30 minutes. Wash the erythrocytes 3 times with a quantity of physiological saline each by centrifugation at 1000 r/min for one minute.

Add 0.2 ml of anti-human globulin serum into each tube, mix well and centrifuge at 1000 r/min for one minute. Observe the results visually. At the same time, negative, positive and erythrocyte control tests shall be performed.

Negative control: Add 0.2 ml of 5% erythrocyte suspensions of groups A and group B, in duplicate, into 0.2 ml of group AB human serum separately and mix well. Carry out the same procedure as that for test sample starting from "allow to stand in 37°C water bath for 30 minutes".

Positive control: Add 0.2 ml of 5% RhD positive group O erythrocyte suspension into 0.2 ml of anti-D IgG serum and mix well. Carry out the same procedure as that for test sample starting from "allow to stand in 37°C water bath for 30 minutes".

Erythrocyte control: Add 0.2 ml of 5% erythrocyte suspension of groups A and B into 0.2 ml of physiological saline separately and mix well. Carry out the same procedure as that for test sample starting from "allow to stand in 37°C water bath for 30 minutes".

Result evaluation

The test is valid if the results of negative and erythrocyte controls are negative and that of positive control is not less than "+++".

The titers of anti-A and anti-B hemagglutinins are calculated as the highest dilution of the test sample which produce agglutination "+", regardless of the volumes of erythrocyte suspension and anti-human globulin serum.

[Note]

(1) The test sample of coagulation factor VIII shall be diluted to 4 IU/ml with physiological saline before test.

(2) Calibration of anti-human globulin serum: Carry out a series of 2-fold dilutions for 0.2 ml of anti-human globulin serum and anti-D serum separately with physiological saline. Add 0.1 ml of 5% packed erythrocytes suspension of RhD-positive group O to each dilution of anti-D serum and mix well. Allow the mixture to stand in 37°C water bath for 30 minutes, wash 3 times separately with physiological saline and then prepare into a 2% cell suspensions. Add 0.2 ml of each dilution of sensitized erythrocytes suspension to a row of diluted anti-human globulin solutions and mix well. Centrifuge at 1000 r/min for one minute and read the result. Repeat the same procedure by replacing the sensitized erythrocytes with an equal volume of un-sensitized human erythrocytes of RhD positive group O as a negative control. If the negative control test is valid, the highest dilution of anti-human globulin serum corresponding to the highest dilution of anti-D serum showing hemagglutination reaction (+) is regarded as the optimal dilution.

(3) The criterion for the judgement of hemagglutination is as follows:

++++: one solid clot;
+++ : several large clots;
++ : medium-sized clots and clear background;
+ : small clots and turbid background;
- : no hemagglutination or hemolysis.

(4) Negative, positive and erythrocyte control tests shall be performed in parallel.

IX K Test for Anticomplement Activity

The method is based on immune hemolytic reaction. The anticomplement activity (ACA) of test sample is determined according to the change of hemolysis rate caused by the consumption of complement.

Reagent

(1) Magnesium and calcium stock solution: Dissolve 1.103 g of calcium chloride and 5.083 g of magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) in water and dilute to 25 ml.

(2) Barbitol buffer stock solution: Dissolve 41.5 g of sodium chloride and 5.1 g of barbitol sodium in 800 ml of water. Adjust pH to 7.3 with 1 mol/L hydrochloric acid. Add 2.5 ml of magnesium and calcium stock solution and dilute to 1000 ml with water. Filter with a 0.22 μm membrane and store at 4°C.

(3) Gelatin-barbitol buffer (GBB) solution: Dissolve 0.625 g of gelatin in 30 ml of water by boiling. Add 100 ml of barbitol buffer stock solution and dilute to 500 ml with water. Prepare fresh solution daily.

(4) Alsever solution: Dissolve 0.5 g of citric acid, 8.0 g of sodium citrate, 20.5 g of glucose and 4.2 g of sodium chloride in water and dilute to 1000 ml (pH about 6.2). Dispense the solution in blood collecting bottles with a quantity needed for one bleeding of a sheep. Carry out steam sterilization at 116°C for 10 minutes (after sterilization, the steam shall be released as soon as possible). Store at 4°C.

(5) Sheep erythrocytes: Collect sheep blood from jugular vein aseptically and mix with an equal volume of Alsever solution. Dispense the mixture in tubes under an aseptic condition and store at 4°C for one week before use.

(6) Hemolysin: Rabbit anti-sheep erythrocyte serum.

(7) Guinea pig serum (complement): Dispense the pooled sera collected from more than ten guinea pigs after removing blood cells by centrifugation at 4°C. Store at -70°C or store lyophilized. The total complement activity of guinea pig serum shall be not less than 200 CH_{50}/ml .

Preparation of 5% sheep erythrocyte suspension

Wash a quantity of above-mentioned sheep

erythrocytes at least 3 times with GBB and suspend in a quantity of the same solution. Add 0.2 ml of the erythrocyte suspension to 2.8 ml of water. Measure the absorbance at 541 nm by ultraviolet-visible spectrophotometry (Appendix II A) after the complete hemolysis of erythrocytes. Adjust the absorbance of the suspension to 0.62 ± 0.01 according to the following formula. After the adjustment, the erythrocyte concentration of the suspension shall be 1×10^9 cells/ml.

Volume (ml) of erythrocyte suspension after dilution = $V_i \times A/0.62$

Where: V_i = Volume of erythrocyte suspension before dilution, ml;

A = Absorbance of hemolyzed erythrocytes before dilution.

Titration of hemolysin

Dilute hemolysin according to the parameters in Table 1. Starting from 1 : 75 dilution of hemolysin, mix 1.0 ml of hemolysin of each dilution with 1.0 ml of 5 % sheep erythrocyte suspension and incubate at 37°C for 30 minutes. To 0.2 ml of each incubated mixture, add 1.10 ml of GBB and 0.2 ml of diluted guinea pig serum (e.g. 1/150 dilution). Incubate at 37°C for 60 minutes. Centrifuge the incubated mixture at 2000 r/min for 5 minutes. Collect the supernatant and measure the absorbance of each tube at 541 nm by ultraviolet-visible spectrophotometry (Appendix II A). The above tests shall be done in duplicate. At the same time, prepare three tubes of non-hemolysis controls by adding 0.1 ml of 5% sheep erythrocytes to 1.4 ml of barbital buffer solution and another three tubes of complete hemolysis controls by adding 0.1 ml of 5% sheep erythrocytes to 1.4 ml of water with the same procedure as mentioned above. Calculate the hemolysis rate (Y) of each tube according to the following formula:

$$Y = \frac{A_s - A_{cn}}{A_{ch} - A_{cn}} \times 100\%$$

Where: Y = Hemolysis rate, %;

A_s = Absorbance of each sample tube;

A_{cn} = Absorbance of non-hemolysis control tube;

A_{ch} = Absorbance of complete hemolysis control tube.

Plot a curve by using hemolysis rate Y as ordinate and dilution factor of hemolysin as abscissas to determine the optimal dilution of the hemolysin for preparation of sensitized sheep erythrocyte. Select a dilution such that further increase in the amount of hemolysin does not cause appreciable change in the degree of hemolysis, and define this dilution as one minimal hemolysin dilution unit (1 MHU) in 1.0 ml. The maximum hemolysis rate shall be within the range of 50%-70%, otherwise the test is invalid.

Table 1 Preparation of hemolysin dilutions

Required dilution of hemolysin	Gelatin-barbital buffer solution, ml	Preparation	
		Hemolysin	
		Dilution(1: x)	ml
7.5	0.65	Undiluted	0.1
10	0.90	Undiluted	0.1
75	1.80	7.5	0.2
100	1.80	10	0.2
150	1.00	75	1.0
200	1.00	100	1.0
300	1.00	150	1.0
400	1.00	200	1.0
600	1.00	300	1.0
800	1.00	400	1.0
1200	1.00	600	1.0
1600	1.00	800	1.0
2400	1.00	1200	1.0
3200*	1.00	1600	1.0
4800*	1.00	2400	1.0

* discard 1.0 ml of the mixture

Preparation of optimal sensitized sheep erythrocytes (EA)

Add a quantity of 2 MHU/ml hemolysin slowly to an equal volume of 5% sheep erythrocyte suspension. Allow the mixture to stand at 37°C for 15 minutes. Store at 2-8°C and use within 6 hours.

Table 2 Protocol for the titration of complement activity

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
GBB(ml)	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	1.3	1.3
Diluted complement(ml)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	—	—
EA(ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Incubate at 37°C for 60 minutes → cool in an ice bath → centrifuge at 2000 r/min for 5 minutes → measure the absorbance of supernatant at 541 nm

Titration of complement activity in guinea pig serum

Dilute the guinea pig serum properly with gelatin barbital buffer solution and titrate the complement according to the protocol in Table 2. A linear regression formula is obtained by regressing the

logarithm of the amount of complement used with the logarithm of $Y/(1-Y)$. Calculate the intercept (a), slope (b) and correlation coefficient (r). The complement activity is calculated by the following formula:

Complement activity (CH_{50}/ml) = $(1/X) \times F/5$
 Where: $(1/X)$ = Reciprocal of antilog a;
 F = Dilution factor of complement

Determination of anticomplement activity (ACA)

According to the titrated guinea pig serum complement activity, dilute the complement to 100 CH_{50}/ml with gelatin barbital solution. Prepare incubation mixtures according to the parameters in Table 3.

The concentration of IVIG in Table 3 is calculated on the basis of 50 mg/ml. If the concentration of IVIG is not 50 mg per ml, the volume of IVIG needed is adjusted by the following formula:

$$\text{Volume of IVIG needed (ml)} = 10 \text{ mg}/C$$

Where: C = IgG (mg) content in 1 ml of IVIG

Table 3 Preparation of incubation mixture

Tube	Test sample (ml)	Complement control (ml)
Test sample (50 mg/ml)	0.2	—
Gelatin-barbital buffer (ml)	0.6	0.8
Complement (100 CH_{50}/ml)	0.2	0.2

Then, calculate the quantity of gelatin barbital buffer solution to be added according to the actual amount of IVIG, but the total volume of the mixture shall be maintained at 0.8 ml. Allow the mixture to stand at 37°C for 60 minutes. To 0.2 ml of the incubated mixture, add 9.8 ml of gelatin-barbital buffer solution (50-fold dilution) and determine the remaining complement activity. The anticomplement activity of test sample is calculated by the following formula. The test is valid if the remaining complement activity of the control is within the range of 80-120 CH_{50}/ml .

$$\text{Anticomplement activity of test sample (\%)} = (D-G)/D \times 100\%$$

Where: D = Remaining complement activity of control, CH_{50}/ml ;

G = Remaining complement activity of test sample, CH_{50}/ml ;

[Note]

- (1) Leukocytes must be removed during the washing of erythrocytes;
- (2) Shake the erythrocyte gently during sensitization.
- (3) Only clarified gel solution is permitted to use.

IX L Determination of Residual Murine IgG

The residual murine IgG content in the recombinant product, after purification by affinity chromatography with monoclonal antibody, is determined by ELISA.

Reagent

- (1) Coating solution (carbonate buffer solution,

pH 9.6): Dissolve 0.32 g of sodium carbonate and 0.586 g of sodium hydrogen carbonate in water and dilute to 200 ml.

(2) PBS (pH 7.4) solution: Dissolve 8.0 g of sodium chloride, 0.20 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.

(3) Washing solution (PBS-Tween 20): Dilute 0.5 ml of polysorbate 20 to 1000 ml with PBS.

(4) Diluent: Dissolve 0.5 g of bovine serum albumin in washing solution and dilute to 100 ml.

(5) Substrate buffer solution (citric acid-PBS): Dissolve 1.84 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 0.51 g of citric acid in water and dilute to 100 ml.

(6) Substrate solution: Dissolve 8 mg of α -phenylenediamine in 20 ml of substrate buffer solution. Add 30 μl of 30% hydrogen peroxide. Prepare just before use.

Preparation of reference solution

Measure accurately a volume of murine IgG reference substance, reconstituted with a quantity of water according to the instructions, and dilute with diluent to contain 100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng and 3.13 ng per ml respectively.

Preparation of test sample solution

Dilute a quantity of test sample to make 1 ml containing one dose of final product. If specifications of the product are not defined, the test sample is diluted to a concentration of the maximum dose of final product per ml.

Procedure

Dilute goat anti-mouse IgG with coating solution to contain 10 μg per ml and add to a 96-well microtitre plate, 100 μl per well. Allow the plate to stand at 4°C overnight (16-18 hours). Wash the plate 3 times with washing solution. Prepare a 1% bovine serum albumin solution with washing solution and add to the plate, 200 μl per well. Block at 37°C for 2 hours. Wash the blocked plate 3 times with washing solution. Add reference and test sample solutions to the plate separately, 100 μl per well, allow to stand at 37°C for one hour. Wash the plate 3 times with washing solution. Dilute horseradish peroxidase-labelled sheep antimouse IgG with diluent following the instructions and add to the plate, 100 μl per well. Allow the plate to stand at 37°C for 30 minutes and wash 3 times with washing solution. Add substrate solution to the plate, 50 μl per well, allow to stand at 37°C for 20 minutes, protected from light. Add 50 μl of stopping solution (1 mol/L sulfuric acid) into each well to stop the reaction. Read the absorbance of each well at 492 nm with microtitre plate reader and analyze the data with a computer software or calculate the result manually with graphic method. Plot a curve with the concentrations of the

reference solutions against their absorbance. The correlation coefficient of the curve shall be more than 0.995. Find out the murine IgG content of test sample solution on the curve through its absorbance. Calculate the residual murine IgG content of test sample by the following formula:

Residual murine IgG content (ng) = $C \times D \times F / T$

Where: C = Murine IgG content of test sample solution, ng/ml;

D = Dilution factor of test sample solution;

F = Specification of final product, IU/dose or $\mu\text{g}/\text{dose}$;

T = Potency or protein content of principal constituent of the test sample, IU/ml or $\mu\text{g}/\text{ml}$.

IX M Test for Reverse Transcriptase Activity

The reverse transcriptase activity in test sample is determined by amplifying a specified fragment with RT-PCR method using brome mosaic virus (BMV) RNA as a template and detecting the hybridization signal of the fragment with probe by ELISA.

Reagent

(1) Diluent for test sample (solution A): Each liter of solution A contains 25 mmol of trishydroxymethylaminomethane-hydrochloric acid (Tris-HCl, pH 7.5), 50 mmol of potassium chloride, 5 mmol of dithiothreitol (DTT), 0.25 mmol of tetramethyl ethylene diamine (EDTA), 25 ml of Triton X-100 and 500 ml of glycerol.

(2) Preservative solution for test sample (solution B): Add 1 mg of leupeptin, 0.7 mg of gastric inhibitory peptide and 1 mg of aprotinin to 1 L of solution A.

(3) Sequences of primer and probe

Upstream primer: 5'-biotin- CGT GGT TGA CAC GCA GAC CTC TTA C-3'

Downstream primer: 5'-TCA ACA CTG TAC GGC ACC CGC ATT C-3'

Probe: 5'-NH₂ ATTATCTGGCC TTT GAG AGTTA CTC TTT G-3'

(4) Template: BMV RNA

(5) Reverse transcription buffer solution: Each liter of reverse transcription buffer solution contains 50 mmol of Tris-HCl (pH 8.3), 40 mmol of potassium chloride, 6 mmol of magnesium chloride, 10 mmol of DTT, 200 μmol of deoxyribonucleotide and 0.8×10^{-3} mmol of downstream primer.

(6) Amplification buffer solution: Each liter of amplification buffer solution contains 25 mmol of Tris-HCl (pH 8.8), 40 mmol of potassium chloride, 2 mmol of magnesium chloride, 200 μmol of deoxyadenosine triphosphate (dATP), 200 μmol

of deoxycytidine triphosphate (dCTP), 200 μmol of deoxyguanosine triphosphate (dGTP), 200 μmol of deoxyuridine triphosphate (dUTP), 0.4×10^{-3} mmol of upstream primer, 0.4×10^{-3} mmol of downstream primer, 0.8 g of ribonuclease A, 6×10^4 U of Taq DNA polymerase and 4×10^4 U of uracil N-glycosylase (UNG).

(7) Blocking solution: PBS containing 3% bovine serum albumin or 0.5% casein.

(8) Denaturation buffer solution: 0.2 mol/L sodium hydroxide, 5 mmol/L EDTA solution.

(9) Coating solution: 0.05 mol/L disodium hydrogen phosphate solution (pH 8.5).

Preparation of test sample, positive control and sensitivity control

(1) Test sample: Centrifuge 200 μl of test sample at 5000 r/min for 5 minutes and collect the supernatant. To 100 μl of the supernatant, add 100 μl of solution B and 2 μl of diethylpyrocarbonate (DEPC)-treated 5% Triton X-100. Mix well and allow the mixture to stand in an ice bath for 15 minutes. Store at -70°C for use.

(2) Positive control: Treat the supernatant of SP 2/0 cell culture by the same procedure as that for test sample and dispense it according to the amount used in a single test. Store at -70°C for use.

(3) Sensitivity control: Dilute a quantity of murine leukemia virus reverse transcriptase (M-MLV) serially with solution A to contain 10^{-8} U per μl . Vortex thoroughly for each dilution. Dispense the mixture according to the amount used in a single test. Store at -70°C for use.

Procedure

(1) Reverse transcription: Make the treated test sample and positive control 10-fold dilution with solution A.

Add 20.8 μl of reverse transcription buffer solution and 0.2 μl of 500 mg/L BMV-RNA into a reverse transcription tube. Mix well and mark. Allow the tube to stand at 70°C for 10 minutes and then set in an ice bath.

Add 4 μl of either diluted test sample, positive control or sensitivity control into corresponding reverse transcription tubes, using solution A as a negative control. The total volume of reverse transcription reaction system is 25 μl . React at 37°C for 90 minutes.

(2) PCR amplification: Add 5 μl of reverse transcription product into 20 μl of PCR amplification buffer solution and mix well. The total volume of PCR amplification reaction system is 25 μl . Perform PCR amplification by the following schedule: 37°C for 30 minutes for reverse transcription, 94°C for 5 minutes for pre-denature, 35 cycles of 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 45 seconds, with a final extension at 72°C for 5 minutes.

(3) Determination of hybridization: Coat DNA binding plate with probe diluted with coating solution properly, 100 μl per well, allow to stand at 4°C overnight. Block the plate with blocking

solution, 200 μ l per well, at 37°C for 60 minutes and wash 3 times with washing solution for use. After amplification, add 25 μ l of denaturation buffer solution into each reaction tube and mix well. Add 25 μ l of test sample and 100 μ l of hybridization solution into each well of the coated plate after blocking and washing. Mix well and react at 37°C for 60 minutes. Wash the plate 5 times with washing solution, add 100 μ l of streptavidin-labelled horseradish peroxidase and react at 37°C for 30 minutes. Wash 5 times with washing solution. Add 100 μ l of colour developing solution into each well and develop at 37°C for 10 minutes. Add 100 μ l of stopping solution into each well to stop the reaction. Read the absorbance of each well with microtitre plate reader at 492 nm using 630 nm as a reference wavelength.

Result evaluation

The cutoff value is defined as the absorbance of negative control plus 0.2.

The absorbance of negative control shall be not more than 0.2. The absorbance less than 0.05 shall be calculated as 0.05.

The result of positive control shall be positive, and its absorbance shall be not less than 1.5.

The result of sensitivity control shall be positive, and its absorbance shall be not less than 0.8.

If the absorbance of test sample is more than the cutoff value, the result shall be judged as positive.

Precaution

(1) If the test sample is cell culture, it shall be subcultured for 3-4 days to form a monolayer. Collect the supernatant for the test. Prior to the test sample taking, the medium shall not be changed.

(2) All the reagents and pipette tips used for the test shall be sterilized. The reagents and materials involving in RNA procedure shall be treated with diethylpyrocarbonate (DEPC).

(3) The areas in which test sample loading, amplification and determination of PCR product are performed, as well as the pipettes and clothes, shall be dedicated.

(4) All the areas shall be disinfected periodically. The PCR product and the pipette tips used for test sample shall be effectively treated in time.

IX N Test for Human Thrombin Activity

The method is based on the principle that thrombin can coagulate human fibrinogen. The thrombin activity of test sample is determined by the formation of coagulum after the test sample is mixed with human fibrinogen.

Reagent

(1) 0.5% fibrinogen solution; Dilute the reconstituted freeze-dried human fibrinogen solution with

physiological saline to a concentration of 5 mg per ml.

(2) Human thrombin solution; Dilute the reconstituted freeze-dried human thrombin with physiological saline to a concentration of 0.5 IU per ml.

Procedure

To 0.2 ml of test sample, add 0.2 ml of 0.5% fibrinogen solution, allow to stand at 37°C for 24 hours. Observe whether coagulum or fibrin is formed at least 2 times during the observation period. Negative and positive control tests shall be performed in parallel.

(1) Negative control; Repeat the above-mentioned procedure using 0.2 ml of physiological saline instead of test sample.

(2) Positive control; Repeat the above-mentioned procedure using 0.2 ml of 0.5 IU/ml human thrombin solution instead of test sample.

Result evaluation

The test is valid if coagulum or fibrin is formed in positive control but not in negative control. No coagulum or fibrin shall be observed visually in the test sample.

[Note]

If the test sample contains heparin, a quantity of protamine sulfate (10 μ g for 1 IU of heparin) shall be added to neutralize the heparin before test.

IX O Test for Activated Coagulation Factor Activity

The method is based on the principle that, in the presence of cephalin, activated coagulation factor can coagulate platelet-poor human plasma. The presence or absence of activated coagulation factor in test sample is determined by the coagulation time after the test sample is mixed with platelet-poor human plasma and cephalin.

Reagent

(1) Platelet-poor human plasma; Collect human blood aseptically and mix well with 3.8% sodium citrate as an anticoagulant at a volume ratio of 9 : 1. Centrifuge at 1500 r/min, 4°C for 30 minutes. Collect the upper two-third of plasma with a plastic syringe and centrifuge at 3500 r/min, 4°C for 30 minutes. Collect the upper two-third of plasma and fill into plastic tubes, 3 ml per tube. Store at -40°C.

(2) Tris buffer solution (pH 7.5); Dissolve 7.27 g of Tris and 5.27 g of sodium chloride in water and dilute to 1000 ml. Adjust pH to 7.5 with hydrochloric acid.

(3) Cephalin suspension; Reconstitute freeze-dried cephalin with water and dilute with a quantity of physiological saline to a concentration so as to make the blank coagulation time between 200 and 300 seconds.

(4) 0.025 mol/L calcium chloride solution: Dissolve 147 g of calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in 1000 ml of water to prepare 1 mol/L calcium chloride stock solution. Make the stock solution 40-fold dilution with water just before use.

(5) Protamine sulfate solution: Dissolve a quantity of protamine sulfate in Tris buffer solution (pH 7.5) and dilute to a suitable concentration.

Preparation of test sample solution

Add a quantity of protamine sulfate into the reconstituted test sample according to the heparin content determined by the method in Appendix IX P to neutralize the heparin (1 IU of heparin shall be neutralized with 10 μg of protamine sulfate), and make the mixture 10-fold and 100-fold dilutions with Tris buffer solution (pH 7.5).

Procedure

Mix 0.1 ml of platelet-poor human plasma with 0.1 ml of cephalin suspension and allow the mixture to stand at 37°C for one minute. Add 0.1 ml of test sample (10-fold or 100-fold dilution) and 0.1 ml of 0.025 mol/L calcium chloride solution preheated to 37°C. Record the coagulation time. A blank control test is performed by the above-mentioned procedure using 0.1 ml of Tris buffer solution (pH 7.5) instead of test sample.

Result evaluation

The test is valid if the coagulation time of blank control is not less than 200 seconds. The coagulation times of 1 : 10 and 1 : 100 test sample dilutions shall be not less than 150 seconds.

[Note]

- (1) The apparatus directly contacting blood or plasma shall be made of plastic or siliconized glass. The test shall be completed within 30 minutes starting from the dilution of test sample.
- (2) Each dilution of the test sample is tested in duplicate.

IX P Determination of Heparin Content (Coagulation Method)

The heparin content in test sample is determined based on the principle that protamine sulfate can neutralize anticoagulant heparin and influence the coagulation time of plasma.

Reagent

- (1) Platelet-poor human plasma: Collect human blood aseptically and mix well with 3.8% sodium citrate anticoagulant at a volume ratio of 9 : 1. Centrifuge at 1500 r/min, 4°C for 30 minutes. Collect the upper two-third of the plasma with a plastic syringe and centrifuge at 3500 r/min, 4°C for 30 minutes. Collect the upper two-third of the

plasma and fill into plastic tubes, 3 ml per tube. Store at -40°C.

(2) Tris buffer solution, pH 7.5: Dissolve 7.27 g of Tris and 5.27 g of sodium chloride in water and dilute to 1000 ml. Adjust pH to 7.5 with hydrochloric acid.

(3) Cephalin suspension: Reconstitute freeze-dried cephalin with water and dilute with a quantity of physiological saline to a concentration so as to make the blank coagulation time between 200 and 300 seconds.

(4) 0.025 mol/L calcium chloride solution: Dissolve 147 g of calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in 1000 ml of water to prepare a 1 mol/L calcium chloride stock solution. Make the stock solution 40-fold dilution with water just before use.

(5) Protamine sulfate solution: Dissolve a quantity of protamine sulfate in Tris buffer solution (pH 7.5) and dilute to a concentration of 1-20 mg per ml.

Preparation of test sample solution

Reconstitute the test sample according to the labelled volume. Transfer 0.5 ml of the reconstituted sample to each of plastic tubes containing 10 μl of protamine sulfate solution at different concentrations respectively and mix well.

Procedure

Add 0.1 ml of cephalin suspension into a plastic tube containing 0.1 ml of platelet-poor human plasma and mix well. Allow the tube to stand at 37°C for one minute. Add 0.1 ml of test sample solution and 0.1 ml of 0.025 mol/L calcium chloride solution preheated to 37°C. Record the coagulation time. A blank control test is performed by the above-mentioned procedure using 0.1 ml of Tris buffer solution (pH 7.5) instead of test sample. The test is valid if the coagulation time of blank control is not less than 200 seconds. The protamine sulfate content in the tube with the shortest coagulation time shall be selected for the neutralization of heparin content in 0.5 ml of the test sample. On this basis, 1 IU heparin can be neutralized by 10 μg of protamine sulfate. For example, if the test sample tube with the shortest coagulation time contains 30 μg of protamine sulfate, 3 IU of heparin in 0.5 ml of test sample is then neutralized. So the heparin content in 1 ml of test sample shall be 6 IU.

[Note]

The apparatus directly contacting blood or plasma shall be made of plastic or siliconized glass.

IX Q Determination of Human Erythrocyte Antibody (Microtitre Plate Method)

The method is based on the principle that

erythrocytes bind to the corresponding antibody with the occurrence of coagulation. The potency of human erythrocyte antibody in test sample is determined by comparing the hemagglutination reaction end points of test sample and control.

Reagent

1% group O erythrocyte suspension; Mix group O blood containing an anticoagulant, from three healthy donors or more, and use within 7 days after blood collection. Wash the blood 3 times with physiological saline before use. The last washing is carried out by centrifugation at 2000 r/min for 10 minutes. Dilute the erythrocyte sediment with physiological saline to 1% for use.

Procedure

Make the test sample a series of 2-fold dilutions on a V-shaped 96-well microtitre plate, with an angle of 90° at the bottom, in duplicate in two rows, 50 µl per well. Add 50 µl of 1% O group erythrocyte suspension into each well and mix well by patting the microtitre plate slightly for 30 seconds. Allow the plate to stand at room temperature for 3 hours and observe the result. A negative control test is performed by the above-mentioned procedure in parallel using physiological saline instead of test sample.

Result evaluation

Put the microtitre plate on a white background and compare the results of test sample wells with those of control wells. If erythrocytes settle at the bottom of the well and form a regular spot without sticking on the wall, the result shall be judged as negative. However, if the wall of the well is stuck with a layer of erythrocytes evenly or only a part of the erythrocytes settle at the bottom and the rest are stuck on the wall, the result shall be judged as positive. The highest dilution of test sample showing positive result is served as the potency of erythrocyte antibody. If the difference between results of test sample of the same batch in the two rows is more than one dilution, the test shall be repeated. If the difference is one dilution, the highest dilution of test sample showing positive result in the two rows is served as the potency of erythrocyte antibody.

IX R Determination of Human Platelet Antibody

The method is based on the principle that platelet bind to the corresponding antibody with the occurrence of coagulation. The potency of human platelet antibody in test sample is determined by comparing the hemagglutination reaction end points of test sample and control.

Reagent

(1) 5% ethylenediaminetetraacetic acid (EDTA)

disodium solution as an anticoagulant; Dissolve 0.365 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 0.875 g of potassium dihydrogen phosphate, 2.125 g of sodium chloride and 12.5 g of ethylenediaminetetraacetic acid disodium ($\text{EDTA-Na}_2 \cdot 2\text{H}_2\text{O}$) in water and dilute to 250 ml.

(2) 0.33% EDTA solution; Dissolve 0.73 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 1.75 g of potassium dihydrogen phosphate, 4.25 g of sodium chloride and 1.65 g of ethylenediaminetetraacetic acid disodium ($\text{EDTA-Na}_2 \cdot 2\text{H}_2\text{O}$) in water and dilute to 500 ml.

(3) Diluent for platelet; Mix group AB sera from more than three donors and inactivate at 56°C for 30 minutes. Add 50 g of barium sulfate into each 100 ml of group AB serum and adsorb at 37°C for one hour. Stir the mixture during adsorption from time to time. Centrifuge the mixture at 3000 r/min for 30 minutes. Discard the precipitate and collect the supernatant for use.

Dilute one volume of the supernatant with three volumes of physiological saline to prepare a diluent for platelet on the date of the test. Care shall be taken to prevent the existence of erythrocytes and the occurrence of hemolysis in the group AB serum.

(4) Preparation of platelet suspension; Collect 20 ml of human intravenous blood and mix with 5% EDTA solution at a volume ratio of 9 : 1. Centrifuge the mixture at 20°C, 800 r/min for 15 minutes and collect the supernatant (plasma). Add 0.33% EDTA solution into the plasma up to the original whole blood volume and centrifuge at 20°C, 1500 r/min for 10 minutes. Discard the supernatant. Wash the sediment with 0.33% EDTA solution twice further by the above-mentioned steps and discard the supernatant. Add 0.5 ml of diluent for platelet into the sediment and mix well. Count platelet and adjust its concentration to 2.5×10^3 – 3.5×10^5 platelets/mm³. (Caution: Allow the platelet suspension to stand on the counting plate for 2-3 minutes at first and the counting shall be completed within 10 minutes.)

Preparation of the test sample solution

Make the test sample a series of 2-fold dilutions with physiological saline to a dilution of 1 : 16.

Preparation of positive control solution

Inactivate 0.5 ml of porcine plasma (or rabbit serum) immunized with human platelets at 60°C for 10 minutes and adsorb with 0.05 g of barium sulfate at 37°C for 15 minutes. Centrifuge at 3000 r/min for 20 minutes and collect the supernatant for use.

Negative control solution

Physiological saline and diluent for platelet.

Procedure

To 0.1 ml of test sample of each dilution, add 0.1 ml of platelet suspension and warm at 37°C for

30 minutes. Drop the mixture onto a counting plate, allow to stand for 2-3 minutes and count under a microscope with a magnifying power of 20-40. Negative and positive controls shall be set up at the same time.

(1) Positive control: To 0.1 ml of positive control solution, add 0.1 ml of platelet suspension and proceed with the same procedure as that for test sample.

(2) Negative control: To 0.1 ml of negative control solution, add 0.1 ml of platelet suspension and proceed with the same procedure as that for test sample.

Result evaluation

The test is valid if positive control shows “++” while negative control shows “-”. The highest dilution of test sample when “+” appears is served as the potency of platelet antibody.

[Note]

(1) —: No coagulum or occasionally two to three platelets appear in a chain array.

(2) +: Small coagula consisting of three to five platelets with a few of free platelets appear.

(3) ++: Large coagula consisting of more than six platelets with few of free platelets appear.

Appendix X

X A *In vitro* Test for Relative Potency of Recombinant Hepatitis B Vaccine (Yeast)

The HBsAg content in test sample is determined by ELISA, and its relative potency is calculated by double parallel line assay against a reference.

Reagent

(1) PBS (pH 7.2): Dissolve 8.850 g of sodium chloride, 0.226 g of sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and 1.698 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) in a quantity of water, adjust pH to 7.2 and dilute with water to 1000 ml.

(2) Treatment solution for test sample: Mix 1.25 ml of 20% diethylaminoethanol and 0.20 ml of 10% Triton X-100 solution with 8.55 ml of PBS thoroughly for use.

(3) Diluent for test sample: Dissolve 10.0 g of bovine serum albumin in PBS and dilute to 1000 ml.

Preparation of reference and test sample solutions

Measure accurately 0.1 ml of reference and test sample, and transfer to two test tubes containing 0.1 ml of treatment solution for test sample respectively. Lid a cover and mix well, allow to stand at 20-28°C for 30-35 minutes. Dilute properly the treated reference and test sample with the diluent and carry out the tests on the dilutions of 1:2000, 1:4000, 1:8000, 1:16000 and 1:32000 or other suitable dilutions, in duplicate. Use the diluent for test sample as a negative control. No dilution is needed for positive and negative controls.

Procedure

The test is carried out according to the instructions of reagent kit. The same tests shall be repeated twice within 3 days. The test is valid if both the mean absorbance of negative and positive controls are within the range specified in the instructions of the reagent kit. Calculate the relative potency by dose-response parallel line assay (see Statistical Methods for Biological Assays described in Chinese Pharmacopoeia, Volume II) with the data obtained from the three tests. The geometric mean obtained from the three calculations is defined as

the *in vitro* relative potency of the test sample. Using the reference as a standard, the test result shall be judged as qualified if the geometric mean of relative potency of test sample is not less than 0.5.

X B *In vivo* Test for Biological Activity of Recombinant Human Erythropoietin (Reticulocyte Method)

This method is based on the stimulating effect of human erythropoietin (EPO) on the production of reticulocyte. The number of reticulocytes in mice injected *s.c.* with EPO increases with the increasing dosage. The *in vivo* biological activity of EPO is determined by dose-response parallel line analysis on the count ratio of reticulocyte to erythrocyte.

Reagent

(1) Dipotassium ethylenediamine tetraacetate (EDTA-K_2) anticoagulant: Dissolve 100 mg of EDTA-K_2 in 10 ml of physiological saline and mix well. Prepare just before use.

(2) Diluent: Dissolve 0.1 g of bovine serum albumin in physiological saline and dilute to 100 ml.

Preparation of standard solution

Reconstitute EPO standard following the instructions and dilute with diluent to three (high, medium and low) concentrations.

Preparation of test sample solution

Dilute the test sample with diluent to three concentrations similar to those of standard solution.

Procedure

Inject *s.c.* the standard and test sample solutions of low, medium and high dosages (e.g. 10 IU, 20 IU and 40 IU per mouse) into the inbred mice (female BALB/c mice) aged 6-8 weeks or B6D2F1 mice respectively. Both the standard and test sample at each dosage are injected into at least four mice, not more than 0.5 ml per mouse. Bleed three to four drops of blood from orbit of each mouse on the 4th day and transfer to a tube containing 200 μl of EDTA-K_2 anticoagulant for determining the cell number ratio (Ret %) of reticulocyte to erythrocyte of each mouse by an

automatic reticulocyte analyzer. Calculate the in vivo biological activity of test sample by dose-response parallel line assay (see Statistical Methods for Biological Assays described in Chinese Pharmacopoeia, Volume II) using injecting dose (IU) against Ret%.

X C Biological Activity Test for Interferon (Cytopathic Inhibition Assay)

The method is based on the principle that interferon can protect human amnion-derived WISH cell from the damage of vesicular stomatitis virus (VSV). The biological activity of interferon is determined by the protective effect curve of live WISH cells after being stained with crystal violet.

Reagent

- (1) MEM or RPMI 1640 medium: Dissolve one bag (the specification is 1 L) of MEM or RPMI 1640 medium powder in water and dilute to 1000 ml. Add 10^5 IU of penicillin, 10^5 IU of streptomycin and 2.1 g of sodium bicarbonate. Mix well after dissolution and sterilize by filtration. Store at 4°C.
- (2) Complete medium: To 10 ml of newborn calf serum, add 90 ml of MEM or RPMI 1640 medium. Store at 4°C.
- (3) Testing medium: Mix 7 ml of newborn calf serum with 93 ml of MEM or RPMI 1640 medium. Store at 4°C.
- (4) Challenge medium: Mix 3 ml of newborn calf serum with 97 ml of MEM or RPMI 1640 medium. Store at 4°C.
- (5) Digestion solution: Dissolve 0.2 g of ethylenediaminetetraacetic acid disodium, 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.152 g of disodium hydrogen phosphate and 0.2 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.
- (6) Staining solution: Dissolve 50 mg of crystal violet in 20 ml of absolute ethanol and dilute to 100 ml with water.
- (7) Destaining solution: Dilute 50 ml of absolute ethanol and 0.1 ml of acetic acid to 100 ml with water.
- (8) PBS: Dissolve 8.0 g of sodium chloride, 0.20 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.

Preparation of standard solution

Reconstitute the national standard for testing the biological activity of human interferon following the instructions and dilute to a concentration of 1000 IU per ml with testing medium, then make eight 4-fold dilutions serially on a 96-well cell culture plate, in duplicate. Prepare the solution

under an aseptic condition.

Preparation of test sample solution

Dissolve the test samples based on the labelled volume and dilute to contain about 1000 IU per ml with testing medium, then make eight 4-fold dilutions serially on a 96-well cell culture plate, in duplicate. Prepare the solution under an aseptic condition.

Procedure

Incubate WISH cells in a culture bottle to form a monolayer of anchored cells. Transfer the cells into complete medium for subculture at a ratio of 1:2-1:4, 2-3 times a week. Discard the medium, wash the cells twice with PBS and digest with digestion solution. Collect the cells and dilute with complete medium to prepare a suspension at a concentration of 2.5×10^5 - 3.5×10^5 cells per ml. Add the suspension onto a 96-well cell culture plate, 100 μ l per well, and incubate at 37°C in a 5% carbon dioxide incubator for 4-6 hours. Transfer the prepared standard and test sample solutions to the plate inoculated with WISH cells, 100 μ l per well and incubate at 37°C in a 5% carbon dioxide incubator for 18-24 hours. Discard the supernatant. Dilute the vesicular stomatitis virus, stored at -70°C, with challenge medium to 100 CCID₅₀, inoculate onto the plate, 100 μ l per well and incubate at 37°C in a 5% carbon dioxide incubator for 24 hours, until the 50% CPE point of standard solution appears at a concentration of 1 IU per ml under microscope. Discard the supernatant, add 50 μ l of staining solution into each well, allow to stand at room temperature for 30 minutes. Remove the staining solution by washing with flowing water carefully and adsorb the residual moisture. Add 100 μ l of destaining solution into each well, allow to stand at room temperature for 3-5 minutes. Mix well and read absorbance with microtitre plate reader at 570 nm using 630 nm as a reference wavelength. Record the determination results.

Analyze the data by using four-parameter regression method or other relevant computer software. Calculate the test result by the following formula:

$$\text{Biological activity (IU/ml) of test sample} = P_r \times [(D_s \times E_s) / (D_r \times E_r)]$$

Where: P_r = Biological activity of standard, IU/ml;

D_s = Pre-dilution factor of test sample;

D_r = Pre-dilution factor of standard;

E_s = Dilution factor of test sample with response equivalent to that of standard at 50% effective concentration;

E_r = Dilution factor of standard with 50% effective concentration

X D Biological Activity Test for Recombinant Human Interleukin -2 (CTLL-2 Cell/MTT Colorimetric Method)

The method is based on the principle that the viability of CTLL-2 cell is dependent on stimulation of interleukin-2 (IL-2) at different potency. The biological activity of IL-2 may be determined according to the growth of CTLL-2 cells.

Reagent

(1) RPMI 1640 medium; Dissolve one bag (the specification is 1L) of RPMI 1640 medium powder in water and dilute to 1000 ml. Add 10^5 IU of penicillin, 10^5 IU of streptomycin and 2.1 g of sodium bicarbonate. Mix well after dissolution and sterilize by filtration. Store at 4°C.

(2) Basic medium; Mix 10 ml of newborn calf serum with 90 ml of RPMI 1640 medium. Store at 4°C.

(3) Complete medium; Add recombinant human IL-2 into 100 ml of basic medium to a final concentration of 400-800 IU. Store at 4°C.

(4) PBS; Dissolve 8.0 g of sodium chloride, 0.20 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.

(5) Thiazole blue (MTT) solution; Dissolve 0.1 g of MTT in PBS and dilute to 20 ml. Sterilize by filtration with a 0.22 μ m membrane. Store at 4°C, protected from light.

(6) Lysis solution; 15% sodium dodecyl sulfate (SDS). Use the solution within 12 months after preparation.

CTLL-2 cell

The CTLL-2 cell culture shall be a weak acidic and slightly turbid liquid. It is used for the biological activity test of interleukin-2 48-60 hours after passage.

Preparation of standard solution

Reconstitute the national standard for the determination of biological activity of human interleukin-2 following the instructions and dilute with basic medium to a concentration of 200 IU per ml, then make eight 2-fold dilutions serially on a 96-well cell culture plate, in duplicate. Keep 50 μ l of standard solution in each well and discard the rest. Prepare the solution under an aseptic condition.

Preparation of test sample solution

Reconstitute the test samples based on the labelled volume, and dilute with basic medium to a concentration of about 200 IU per ml, then make eight 2-fold dilutions serially on a 96-well cell

culture plate, in duplicate. Keep 50 μ l of test sample solution in each well and discard the rest. Prepare the solution under an aseptic condition.

Procedure

Incubate CTLL-2 cells in complete medium at 37°C in a 5% carbon dioxide incubator to prepare enough cells for test. Collect the cells by centrifugation, wash 3 times with RPMI 1640 medium and resuspend at a concentration of 6.0×10^5 cells per ml in basic medium. Keep at 37°C in 5% carbon dioxide incubator for use. Add the cell suspension onto a 96-well cell culture plate containing standard and test sample solutions, 50 μ l per well, and incubate at 37°C in a 5% carbon dioxide incubator for 18-24 hours. Add 20 μ l of MTT solution into each well and incubate the plate at 37°C in a 5% carbon dioxide incubator for 4-6 hours. Add 150 μ l of lysis solution into each well and incubate at 37°C in a 5% carbon dioxide incubator for 18-24 hours. All the above steps shall be carried out under an aseptic condition. Mix the cell culture thoroughly and read absorbance with microtiter plate reader at 570 nm using 630 nm as a reference wavelength. Record the determination results.

Analyze the data by using four-parameter regression method or other relevant computer software. Calculate the test result by the following formula;

$$\text{Biological activity (IU/ml) of test sample} = P_r \times \left[(D_s \times E_s) / (D_r \times E_r) \right]$$

Where: P_r = Biological activity of standard, IU/ml;

D_s = Pre-dilution factor of test sample;

D_r = Pre-dilution factor of standard;

E_s = Dilution factor of test sample with response equivalent to that of standard at 50% effective concentration;

E_r = Dilution factor of standard with 50% effective concentration

X E Biological Activity Test for Recombinant Human Granulocyte Colony-stimulating Factor (NFS-60 Cell Strain/MTT Colorimetric Method)

The method is based on the principle that the growth rate of murine myeloid leukemia cells (NFS-60) is dependent on stimulation of recombinant human granulocyte colony-stimulating factor (rhG-CSF) at different potency. The biological activity of rhG-CSF is determined according to the growth of NFS-60 cells.

Reagent

(1) RPMI 1640 medium; Dissolve one bag (the

specification is 1L) of RPMI 1640 medium powder in water and dilute to 1000 ml. Add 10^5 IU of penicillin, 10^5 IU of streptomycin and 2.1 g of sodium bicarbonate. Mix well after dissolution and sterilize by filtration. Store at 4°C .

(2) Basic medium; Mix 100 ml of newborn calf serum with 900 ml of RPMI 1640 medium. Store at 4°C .

(3) Complete medium; Add rhG-CSF into basic medium to a final concentration of 20 ng or 3000 IU per ml.

(4) PBS; Dissolve 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.

(5) Thiazole blue (MTT) solution; Dissolve 0.10 g of MTT powder in 20 ml of PBS to prepare a 5.0 mg/ml solution. Sterilize by filtration with a $0.22\text{ }\mu\text{m}$ membrane. Store at 4°C , protected from light.

(6) Lysis solution; Dilute 14 ml of hydrochloric acid and 50 ml of Triton X-100 solution to 500 ml with isopropanol. Store at room temperature, protected from light.

Preparation of standard solution

Reconstitute the standard for the determination of biological activity of rhG-CSF following the instructions and dilute with basic medium to a concentration of 50-100 IU per ml, then make eight 2-fold dilutions serially on a 96-well cell culture plate, in duplicate. Keep $50\text{ }\mu\text{l}$ of standard solution in each well and discard the rest. Prepare the solution under an aseptic condition.

Preparation of test sample solution

Dissolve the test samples based on the labelled volume, and dilute with basic medium to a concentration of about 50-100 IU per ml, then make eight 2-fold dilutions serially on a 96-well cell culture plate, in duplicate. Keep $50\text{ }\mu\text{l}$ of test sample solution in each well and discard the rest. Prepare the solution under an aseptic condition.

Procedure

Incubate NFS-60 cell strain in complete medium at 37°C in a 5% carbon dioxide incubator. Control the concentration of culture at a range of 1.0×10^5 - 4.0×10^5 cells per ml. The culture which has been incubated for 24-36 hours after passage shall be used for the biological activity test. Prewarm all the solutions for test to 37°C . Centrifuge enough culture to collect NFS-60 cells. Wash the cells 3 times with RPMI 1640 medium, resuspend at a concentration of 2.0×10^5 cells per ml in basic medium, and keep at 37°C for use. Add the cell suspension onto a 96-well cell culture plate containing standard and test sample solutions, $50\text{ }\mu\text{l}$ per well, and incubate at 37°C in a 5% carbon dioxide incubator for 40-48 hours. Add $20\text{ }\mu\text{l}$ of MTT solution into each well, and incubate the plate at 37°C in a 5% carbon dioxide

incubator for 5 hours. All the above steps shall be carried out under an aseptic condition. Add $100\text{ }\mu\text{l}$ of lysis solution into each well and mix well. Read absorbance with microtitre plate reader at 570 nm using 630 nm as a reference wavelength. Record the determination results.

Analyze the data by using four-parameter regression method or other relevant computer software. Calculate the test result by the following formula:

Biological activity (IU/ml) of test sample =

$$P_r \times [(D_s \times E_s) / (D_r \times E_r)]$$

Where: P_r = Biological activity of standard, IU/ml;

D_s = Pre-dilution factor of test sample;

D_r = Pre-dilution factor of standard;

E_s = Dilution factor of test sample with response equivalent to that of standard at 50% effective concentration;

E_r = Dilution factor of standard with 50% effective concentration

X F Biological Activity Test for Recombinant Human Granulocyte/Macrophage Colony-stimulating Factor (TF-1 Cell/MTT Colorimetric Method)

The method is based on the principle that the growth rate of human erythroleukemia cell (TF-1) is dependent on stimulation of recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF) at different potency. The biological activity of rhGM-CSF is determined according to the growth of TF-1 cells.

Reagent

(1) RPMI 1640 medium; Dissolve one bag (the specification is 1L) of RPMI 1640 medium powder in water and dilute to 1000 ml. Add 10^5 IU of penicillin, 10^5 IU of streptomycin and 2.1 g of sodium bicarbonate, mix well after dissolution and sterilize by filtration. Store at 4°C .

(2) Basic medium; Mix 100 ml of newborn calf serum and 900 ml of RPMI 1640 medium. Store at 4°C .

(3) Complete medium; Add rhGM-CSF into basic medium to a final concentration of 5.0 ng or 80 IU per ml.

(4) PBS; Dissolve 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.

(5) Thiazole blue (MTT) solution; Dissolve 0.10 g of MTT powder in 20 ml of PBS to prepare a 5 mg/ml solution. Sterilize by filtration with a $0.22\text{ }\mu\text{m}$ membrane. Store at 4°C , protected from light.

(6) Lysis solution: Dilute 14 ml of hydrochloric acid and 50 ml of Triton X-100 solution to 500 ml with isopropanol.

Preparation of standard solution

Reconstitute the rhGM-CSF standard following the instructions and dilute with basic medium to a concentration of 10-20 IU per ml. Make eight 2-fold dilutions serially on a 96-well cell culture plate, in duplicate. Keep 50 μ l in each well and discard the rest. Prepare the solution under an aseptic condition.

Preparation of test sample solution

Reconstitute the test samples based on the labelled volume, and dilute with basic medium to a concentration of about 10-20 IU per ml, then make eight 2-fold dilutions serially on a 96-well cell culture plate, in duplicate. Keep 50 μ l in each well and discard the rest. Prepare the solution under an aseptic condition.

Procedure

Incubate TF-1 cell strain in complete medium at 37°C in a 5% carbon dioxide incubator. Control the concentration of culture at a range of 2.0×10^5 - 7.0×10^5 cells per ml. The culture which has been incubated for 24-36 hours after passage shall be used for biological activity test. Prewarm all the solutions for test to 37°C. Centrifuge enough culture to collect TF-1 cells. Wash the cells 3 times with basic medium, resuspend at a concentration of 4.0×10^5 cells per ml in basic medium, and keep at 37°C for use. Add the cell suspension onto a 96-well cell culture plate containing standard and test sample solutions, 50 μ l per well, and incubate at 37°C in a 5% carbon dioxide incubator for 48-52 hours. Add 20 μ l of MTT solution into each well and incubate the plate at 37°C in a 5% carbon dioxide incubator for 5 hours. All the above steps shall be carried out aseptically. Add 100 μ l of lysis solution into each well and mix well. Read absorbance with microtitre plate reader at 570 nm using 630 nm as a reference wavelength. Record the determination results.

Analyze the data by using four-parameter regression method or other relevant computer software. Calculate the test result by the following formula:

$$\text{Biological activity (IU/ml) of test sample} = P_r \times \left[(D_s \times E_s) / (D_r \times E_r) \right]$$

Where: P_r = Biological activity of standard, IU/ml;

D_s = Pre-dilution factor of test sample;

D_r = Pre-dilution factor of standard;

E_s = Dilution factor of test sample with response equivalent to that of standard at 50% effective concentration;

E_r = Dilution factor of standard with 50% effective concentration

X G Biological Activity Test for Recombinant Bovine Basic Fibroblast Growth Factor

(Cell Proliferation/MTT Colorimetric Method)

The method is based on the stimulating effect of recombinant bovine basic fibroblast growth factor (rbBFGF) on the growth of murine embryonic fibroblasts (BALB/c 3T3 cell). The biological activity of rbBFGF is thus determined by the effect on the growth of BALB/c 3T3 cells.

Reagent

(1) RPMI 1640 medium: Dissolve one bag (the specification is 1L) of RPMI 1640 medium powder in water and dilute to 1000 ml. Add 10^5 IU of penicillin, 10^5 IU of streptomycin and 2.1 g of sodium bicarbonate, mix well after dissolution and sterilize by filtration. Store at 4°C.

(2) Maintenance medium: Dilute 4 ml of newborn calf serum to 1000 ml with RPMI 1640 medium.

(3) Complete medium: Dilute 100 ml of newborn calf serum to 1000 ml with RPMI 1640 medium.

(4) PBS: Dissolve 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.

(5) Thiazole blue (MTT) solution: Dissolve 0.10 g of MTT powder in 20 ml of PBS. Sterilize by filtration with a 0.22 μ m membrane. Store at 4°C, protected from light.

Preparation of standard solution

Reconstitute the standard of rbBFGF following the instructions and dilute with maintenance medium to a concentration of 40 IU per ml, then make eight 4-fold dilutions serially on a 96-well cell culture plate, in duplicate. Prepare the solution under an aseptic condition.

Preparation of test sample solution

Reconstitute the test sample based on the labelled volume and dilute with maintenance medium to contain about 40 IU per ml. Make eight 4-fold dilutions serially on a 96-well cell culture plate, in duplicate. Prepare the solution under an aseptic condition.

Procedure

Incubate BALB/c 3T3 cell strain in complete medium at 37°C in a 5% carbon dioxide incubator. Control the concentration of culture at a range of 1.0×10^5 - 5.0×10^5 cells per ml. The culture which has been incubated for 24-36 hours after passage shall be used for the determination of biological activity of rbBFGF. Discard the medium, digest and collect cells to prepare a suspension of 5.0×10^4 - 1.0×10^5 cells per ml with complete medium.

Inoculate the suspension onto a 96-well cell culture plate, 100 μ l per well, and incubate at 37°C in a 5% carbon dioxide incubator for 24 hours. Replace the complete medium with maintenance medium and incubate at 37°C in a 5% carbon dioxide incubator for another 24 hours. Discard the maintenance medium, add standard and test sample solutions, 100 μ l per well, and incubate at 37°C in a 5% carbon dioxide incubator for 64-72 hours. Add 20 μ l of MTT solution into each well and incubate at 37°C in a 5% carbon dioxide incubator for 5 hours. All the above steps shall be carried out under an aseptic condition. Discard the liquid on the plate, add 100 μ l of dimethyl sulfoxide (DMSO) into each well and mix thoroughly. Read the absorbance with microtitre plate reader at 570 nm using 630 nm as a reference wavelength. Record the determination results. Analyze the data by using four-parameter regression method or other relevant computer software. Calculate the test result by the following formula:

Biological activity (IU/ml) of test sample =

$$P_r \times [(D_s \times E_s) / (D_r \times E_r)]$$

Where: P_r = Biological activity of standard, IU/ml;

D_s = Pre-dilution factor of test sample;

D_r = Pre-dilution factor of standard;

E_s = Dilution factor of test sample with response equivalent to that of standard at 50% effective concentration;

E_r = Dilution factor of standard with 50% effective concentration.

X H Biological Activity Test for Recombinant Epidermal Growth Factor (Cell Proliferation/MTT Colorimetric Method)

The method is based on the stimulating effect of recombinant human epidermal growth factor (rhEGF) on the growth of murine embryonic fibroblast (BALB/c 3T3 cell). The biological activity of rhEGF is thus determined by the effect on the growth of BALB/c 3T3 cells.

Reagent

(1) RPMI 1640 medium; Dissolve one bag (the specification is 1L) of RPMI 1640 medium powder in water and dilute to 1000 ml. Add 10^5 IU of penicillin, 10^5 IU of streptomycin and 2.1 g of sodium bicarbonate, mix well after dissolution and sterilize by filtration. Store at 4°C.

(2) Maintenance medium; Dilute 4 ml of newborn calf serum to 1000 ml with RPMI 1640 medium.

(3) Complete medium; Dilute 100 ml of newborn calf serum to 1000 ml with RPMI 1640 medium.

(4) PBS; Dissolve 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium

hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate in water and dilute to 1000 ml. Sterilize at 121°C for 15 minutes.

(5) Thiazole blue (MTT) solution; Dissolve 0.10 g of MTT powder in 20 ml of PBS. Sterilize by filtration with a 0.22 μ m membrane. Store at 4°C, protected from light.

Preparation of standard solution

Reconstitute the standard of rhEGF following the instructions and dilute with maintenance medium to a concentration of 50 IU per ml, then make eight 4-fold dilutions serially on a 96-well cell culture plate, in duplicate. Prepare the solution under an aseptic condition.

Preparation of test sample solution

Reconstitute the test samples based on the labelled volume, and dilute with maintenance medium to a concentration of about 50 IU per ml, then make eight 4-fold dilutions serially on a 96-well cell culture plate, in duplicate. Prepare the solution under an aseptic condition.

Procedure

Incubate BALB/c 3T3 cell strain in complete medium at 37°C in a 5% carbon dioxide incubator. Control the concentration of culture at a range of 1.0×10^5 - 5.0×10^5 cells per ml. The culture which has been incubated for 24-36 hours after passage shall be used for the determination of biological activity of rhEGF. Discard the medium, digest and collect cells to prepare a suspension of 5.0×10^4 - 8.0×10^4 cells per ml with complete medium. Inoculate the suspension onto a 96-well cell culture plate, 100 μ l per well, and incubate at 37°C in a 5% carbon dioxide incubator for 24 hours. Replace the complete medium with maintenance medium and incubate at 37°C in a 5% carbon dioxide incubator for another 24 hours. Discard the maintenance medium, add standard and test sample solutions, 100 μ l per well, and incubate at 37°C in a 5% carbon dioxide incubator for 64-72 hours. Add 20 μ l of MTT solution into each well and incubate at 37°C in a 5% carbon dioxide incubator for 5 hours. All the above steps shall be carried out under an aseptic condition. Discard the liquid on the plate, add 100 μ l of dimethyl sulfoxide (DMSO) into each well and mix thoroughly. Read the absorbance with microtitre plate reader at 570 nm using 630 nm as a reference wavelength. Record the determination results.

Analyze the data by using four-parameter regression method or other relevant computer software. Calculate the test result by the following formula:

Biological activity (IU/ml) of test sample =

$$P_r \times [(D_s \times E_s) / (D_r \times E_r)]$$

Where: P_r = Biological activity of standard, IU/ml;

D_s = Pre-dilution factor of test sample;

D_r = Pre-dilution factor of standard;

E_s = Dilution factor of test sample with response equivalent to that of standard

at 50% effective concentration;
 E_r = Dilution factor of standard with 50% effective concentration

X I Biological Activity Test for Recombinant Streptokinase

The method is based on the principle that a complex formed by streptokinase with plasminogen can activate free plasminogen to become a biologically active plasmin. The biological activity of recombinant streptokinase is determined quantitatively with the transparent rings of lysis formed on agarose plate due to the degradation of human fibrin into soluble fibrin fragments by biologically active plasmin.

Reagent

(1) Human thrombin solution; Dissolve human thrombin and dilute to a concentration of 100 IU per ml with physiological saline solution. Store at -20°C .

(2) Human plasminogen solution; Dissolve human plasminogen with physiological saline and dilute to a concentration of 0.5 mg per ml. Store at -20°C .

(3) Human fibrinogen solution; Prepare just before use. Preheat human fibrinogen and physiological saline in 37°C water bath for 15 minutes before preparation. Dissolve fibrinogen in a quantity of physiological saline. Keep the solution in 37°C water bath for 30 minutes to dissolve the fibrinogen completely and dilute to a concentration of 6 mg per ml with physiological saline.

Preparation of standard solution

Reconstitute the national standard for determination of biological activity of recombinant streptokinase following the instructions and dilute with physiological saline to the concentrations of 1000 IU, 250 IU, 62.5 IU, 15.6 IU and 3.9 IU per ml respectively.

Preparation of test sample solution

Reconstitute the test sample based on the labelled volume, and dilute with physiological saline to a concentration of 100 IU or 1 μg per ml.

Procedure

Swell 125 mg of agarose in 23 ml of physiological saline by boiling, and equilibrate in $55-60^{\circ}\text{C}$ water bath. Add 14 μl of 100 IU per ml human thrombin solution and 280 μl of 0.5 mg/ml human plasminogen solution with shaking, then add 2.2 ml of 6 mg/ml human fibrinogen solution and shake continuously until the mixture is turbid. Pour the mixture into a culture dish 8 cm in diameter immediately and allow the culture dish to stand horizontally. After agarose is coagulated completely, allow the culture dish to stand at 4°C for at least 30 minutes and use within 2 days. Dig wells 2 mm in diameter, on the coagulated agarose

plate containing fibrin. Add test sample and standard solutions at each dilution into the wells separately in duplicate, 10 μl per well, allow the culture dish to stand horizontally at 37°C in a wet box for 24 hours. Measure the longitudinal and transverse diameters of each lysis ring twice separately and calculate the mean diameters. A linear regression formula is obtained by regressing the logarithm of the standard activity of each dilution with the logarithm of mean diameter of the corresponding lysis ring. Calculate the biological activity of test sample by inserting the logarithm of diameter of its lysis ring into the linear regression formula.

X J Potency Test for Human Coagulation Factor II (One-step Method)

The potency of human coagulation factor II is determined by one-step method using human coagulation factor II deficiency plasma as substrate plasma.

Reagent

(1) Diluent; Dissolve 11.75 g of barbital sodium and 14.67 g of sodium chloride in a quantity of water, adjust pH to 7.3 with 1 mol/L hydrochloric acid and dilute to 2000 ml with water. Add 20% human albumin to a final concentration of 1% just before use.

(2) Calcium-containing thromboplastin

(3) Coagulation factor II deficiency plasma; Human plasma or artificial substrate plasma, in which human coagulation factor II concentration is less than 1%.

Preparation of standard solution of human coagulation factor II

Dilute the standard with coagulation factor II deficiency plasma to a concentration of 1 IU per ml, then make 10-fold, 20-fold, 40-fold and 80-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Preparation of test sample solution

Dilute the test sample with coagulation factor II deficiency plasma to a concentration of about 1 IU per ml, then make 10-fold, 20-fold, 40-fold and 80-fold dilutions respectively with diluent, allow to stand in an ice bath for use.

Procedure

Mix 0.1 ml of test sample solution and 0.1 ml of coagulation factor II deficiency human plasma thoroughly and warm in 37°C water bath for a certain time (3 minutes in general). Add 0.2 ml of calcium-containing thromboplastin preheated to 37°C and record the coagulation time.

Repeat the above-mentioned procedure using 0.1 ml of standard solution of human coagulation

factor II at various dilutions instead of the test sample solution.

A linear regression formula is obtained by regressing the logarithm of potency (IU/ml) of human coagulation factor II standard solution with the logarithm of corresponding coagulation time (second). Calculate the potency of human coagulation factor II in test sample solution and multiply with the dilution factor to obtain the human coagulation factor II potency (IU/ml) of test sample.

[Note]

- (1) The correlation coefficient of linear regression shall be not less than 0.98.
- (2) Each dilution of test sample shall be determined in duplicate, and the difference between the two results shall not exceed 10% of the mean value, otherwise the test shall be repeated.
- (3) The apparatus directly contacting the standard, test sample and plasma shall be made of plastic or siliconized glass.
- (4) The test is carried out with an automatic blood coagulation analyzer following the instructions.

X K Potency Test for Human Coagulation Factor VII (One-step Method)

The potency of human coagulation factor VII is determined by one step method using human coagulation factor VII deficiency plasma as substrate plasma.

Reagent

- (1) Diluent; Dissolve 11.75 g of barbitol sodium and 14.67 g of sodium chloride in a quantity of water, adjust pH to 7.3 with 1 mol/L hydrochloric acid and dilute to 2000 ml with water. Add human albumin to a final concentration of 1% just before use.
- (2) Calcium-containing thromboplastin
- (3) Coagulation factor VII deficiency plasma; Human plasma or artificial substrate plasma, in which human coagulation factor VII concentration is less than 1%.

Preparation of standard solution of human coagulation factor VII

Dilute the standard with coagulation factor VII deficiency plasma to a concentration of 1 IU per ml, then make 10-fold, 20-fold, 40-fold and 80-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Preparation of test sample solution

Dilute the test sample with coagulation factor VII deficiency plasma to a concentration of about 1 IU per ml, then make 10-fold, 20-fold and 40-fold dilutions respectively with diluent, allow to stand in an ice bath for use.

Procedure

Mix 0.1 ml of test sample solution and 0.1 ml of coagulation factor VII deficiency plasma thoroughly and warm in 37°C water bath for a certain time (3 minutes in general). Add 0.2 ml of calcium-containing thromboplastin preheated to 37°C and record the coagulation time.

Repeat the above-mentioned procedure using 0.1 ml of standard solution of human coagulation factor VII of various dilutions instead of the test sample solution.

A linear regression formula is obtained by regressing the logarithm of potency (IU/ml) of human coagulation factor VII standard solution with the logarithm of corresponding coagulation time (second). Calculate the potency of human coagulation factor VII in test sample solution and multiply with the dilution factor to obtain the human coagulation factor VII potency (IU/ml) of test sample.

[Note]

- (1) The correlation coefficient of linear regression shall be not less than 0.98.
- (2) Each dilution of test sample shall be determined in duplicate, and the difference between the two results shall not exceed 10% of the mean value, otherwise the test shall be repeated.
- (3) The apparatus directly contacting the standard, test sample and plasma shall be made of plastic or siliconized glass.
- (4) The test is carried out with an automatic blood coagulation analyzer following the instructions.

X L Potency Test for Human Coagulation Factor IX (One-step Method)

The potency of human coagulation factor IX is determined by one-step method using human coagulation factor IX deficiency plasma as substrate plasma.

Reagent

- (1) 3.8% sodium citrate solution; Dissolve 9.5 g of anhydrous sodium citrate in water and dilute to 250 ml.
- (2) Imidazole buffer solution (pH 7.3); Dissolve 0.68 g of imidazole and 1.17 g of sodium chloride in 100 ml of water. Add 42.2 ml of 0.1 mol/L hydrochloric acid solution and dilute to 200 ml with water.
- (3) Diluent; Mix one volume of 3.8% sodium citrate with five volumes of imidazole buffer solution. Add 20% human albumin to a final concentration of 1%.
- (4) Activated partial thromboplastin time (APTT) reagent

(5) Human coagulation factor IX deficiency plasma: Human plasma or artificial substrate plasma, in which human coagulation factor IX concentration is less than 1%.

(6) 0.05 mol/L calcium chloride solution: Dissolve 147 g of calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in water and dilute to 1000 ml to prepare a 1 mol/L calcium chloride stock solution. Make the stock solution 20-fold dilution with water to prepare a 0.05 mol/L calcium chloride solution just before use.

Preparation of standard solution of human coagulation factor IX

Dilute the standard human coagulation factor IX with coagulation factor IX deficiency plasma to a concentration of 1 IU per ml, then make 10-fold, 20-fold, 40-fold and 80-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Preparation of test sample solution

If the test sample contains heparin, the heparin shall be neutralized with protamine sulfate at first. Dilute the test sample with coagulation factor IX deficiency plasma to a concentration of about 1 IU per ml, then make 10-fold, 20-fold and 40-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Procedure

Keep 0.1 ml of APTT reagent in 37°C water bath for a certain time (4 minutes in general), add 0.1 ml of coagulation factor IX-deficiency plasma and 0.1 ml of test sample solution, mix well and warm in 37°C water bath for a certain time (5 minutes in general). Add 0.1 ml of 0.05 mol/L calcium chloride solution preheated to 37°C and record the coagulation time.

Repeat the above-mentioned procedure using 0.1 ml of standard solution of human coagulation factor IX of various dilutions instead of the test sample solution.

A linear regression formula is obtained by regressing the logarithm of potency (IU/ml) of human coagulation factor IX standard solution with the logarithm of corresponding coagulation time (second). Calculate the potency of human coagulation factor IX in test sample solution and multiply with the dilution factor to obtain the human coagulation factor IX potency (IU/ml) of test sample.

[Note]

(1) The correlation coefficient of linear regression shall be not less than 0.98.

(2) Each dilution of test sample shall be determined in duplicate, and the difference between the two results shall not exceed 10% of the mean result, otherwise the test shall be repeated.

(3) The apparatus directly contacting the standard, test sample and plasma shall be made of plastic or siliconized glass.

The test is carried out with an automatic blood

coagulation analyzer following the instructions.

X M Potency Test for Human Coagulation Factor X (One-step Method)

The potency of human coagulation factor X is determined by one-step method using human coagulation factor X-deficiency plasma as substrate plasma.

Reagent

(1) Diluent: Dissolve 11.75 g of barbital sodium and 14.67 g of sodium chloride in a quantity of water, adjust pH to 7.3 with 1 mol/L hydrochloric acid and dilute to 2000 ml with water. Add human albumin to a final concentration of 1% just before use.

(2) Calcium-containing thromboplastin

(3) Coagulation factor X deficiency plasma: Human plasma or artificial substrate plasma, in which human coagulation factor X concentration is less than 1%.

Preparation of standard solution of human coagulation factor X

Dilute the standard with coagulation factor X deficiency plasma to a concentration of 1 IU per ml, then make 10-fold, 20-fold, 40-fold and 80-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Preparation of test sample solution

Dilute the test sample with coagulation factor X deficiency plasma to a concentration of about 1 IU per ml, then make 10-fold, 20-fold and 40-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Procedure

Mix 0.1 ml of test sample solution and 0.1 ml of coagulation factor X-deficiency plasma well and warm in 37°C water bath for a certain time (3 minutes in general). Add 0.2 ml of calcium-containing thromboplastin preheated to 37°C and record the coagulation time.

Repeat the above-mentioned procedure using 0.1 ml of standard solution of human coagulation factor X of various dilutions instead of the test sample solution.

A linear regression formula is obtained by regressing the logarithm of potency (IU/ml) of human coagulation factor X standard solution with the logarithm of corresponding coagulation time (second). Calculate the potency of human coagulation factor X in test sample solution and multiply with the dilution factor to obtain the human coagulation factor X potency (IU/ml) of test sample.

[Note]

- (1) The correlation coefficient of linear regression shall be not less than 0.98.
- (2) Each dilution of test sample shall be determined in duplicate, and the difference between the two results shall not exceed 10% of the mean result, otherwise the test shall be repeated.
- (3) The apparatus directly contacting the standard, test sample and plasma shall be made of plastic or siliconized glass.
- (4) The test is carried out with an automatic blood coagulation analyzer following the instructions.

X N Potency Test for Human Coagulation Factor VIII (One-step Method)

The potency of human coagulation factor VIII is determined by one-step method using human coagulation factor VIII deficiency plasma as substrate plasma.

Reagent

- (1) 3.8% sodium citrate solution: Dissolve 9.5 g of anhydrous sodium citrate in water and dilute to 250 ml.
- (2) Imidazole buffer solution (pH 7.3): Dissolve 0.68 g of imidazole and 1.17 g of sodium chloride in water and dilute to 100 ml. Add 42.2 ml of 0.1 mol/L hydrochloric acid solution and dilute to 200 ml with water.
- (3) Diluent: Mix one volume of 3.8% sodium citrate with five volumes of imidazole buffer solution and add 20% human albumin to a final concentration of 1%.
- (4) Activated partial thromboplastin time (APTT) reagent
- (5) Human coagulation factor VIII deficiency plasma: Human plasma or artificial substrate plasma, in which human coagulation factor VIII concentration is less than 1%.
- (6) 0.05 mol/L calcium chloride solution: Dissolve 147 g of calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in water and dilute to 1000 ml to prepare a 1 mol/L calcium chloride stock solution. Dilute the stock solution 20-fold with water to prepare a 0.05 mol/L calcium chloride solution just before use.

Preparation of standard solution of human coagulation factor VIII

Dilute the standard human coagulation factor VIII with coagulation factor VIII deficiency plasma to a concentration of 1 IU per ml. Then, make 10-fold, 20-fold, 40-fold and 80-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Preparation of test sample solution

Dilute the test sample with coagulation factor VIII deficiency plasma to a concentration of about 1 IU

per ml, then make 10-fold, 20-fold and 40-fold dilutions with diluent respectively, allow to stand in an ice bath for use.

Procedure

Keep 0.1 ml of APTT reagent in 37°C water bath for a certain time (4 minutes in general), add 0.1 ml of coagulation factor VIII deficiency plasma and 0.1 ml of test sample solution, mix well and keep warm in 37°C water bath for a certain time (5 minutes in general). Add 0.1 ml of 0.05 mol/L calcium chloride solution preheated to 37°C and record the coagulation time.

Repeat the above-mentioned procedure using 0.1 ml of standard solution of human coagulation factor VIII of various dilutions instead of the test sample solution.

A linear regression formula is obtained by regressing the logarithm of potency (IU/ml) of human coagulation factor VIII standard solution with the logarithm of corresponding coagulation time (second). Calculate the potency of human coagulation factor VIII in test sample solution and multiply with the dilution factor to obtain the human coagulation factor VIII potency (IU/ml) of test sample.

[Note]

- (1) The correlation coefficient of linear regression shall be not less than 0.98.
- (2) Each dilution of test sample shall be determined in duplicate, and the difference between the two results shall not exceed 10% of the mean result, otherwise the test shall be repeated.
- (3) The apparatus directly contacting the standard, test sample and plasma shall be made of plastic or siliconized glass.
- (4) The test is carried out with an automatic blood coagulation analyzer following the instructions.

X O Potency Test for Diphtheria Antibody in Human Immunoglobulin

The method is based on the principle that sheep erythrocytes after treatment with aldehyde and tannic acid can adsorb diphtheria toxoid onto their surfaces. The adsorbed toxoid can bind the diphtheria antitoxin in test sample and cause specific agglutination. The potency of diphtheria antitoxin of test sample is determined by comparison of the agglutination reaction end points.

Reagent

- (1) Physiological saline containing 1% rabbit serum: Collect rabbit blood aseptically, separate serum and inactivate at 56°C for 30 minutes. Mix 0.5 ml of the rabbit serum with 49.5 ml of physiological saline thoroughly.
- (2) Diphtheria antitoxin diagnostic erythrocyte

suspension; Reconstitute freeze-dried diphtheria antitoxin diagnostic erythrocyte with physiological saline containing 1% rabbit serum to prepare a 5% erythrocyte suspension.

Preparation of diphtheria antitoxin standard solution

Dilute the diphtheria antitoxin standard with physiological saline containing 1% rabbit serum to a concentration of 0.2 HAU per ml.

Preparation of test sample solution

Make the test sample 4-fold dilution with physiological saline containing 1% rabbit serum.

Procedure

Make the test sample 2-fold dilutions serially with physiological saline containing 1% rabbit serum on a UV type microtitre plate and keep 25 μ l of solution in each well. Add 25 μ l of diphtheria antitoxin diagnostic erythrocyte suspension into each well and mix thoroughly on an oscillator for 30-60 seconds. Bind at 37°C in a wet box for one hour.

Make diphtheria antitoxin standard solution 2-fold dilutions serially with physiological saline containing 1% rabbit serum on a UV type microtitre plate and keep 25 μ l of solution in each well. Carry out the same procedure as that for test sample starting from adding 25 μ l of diphtheria antitoxin diagnostic erythrocyte suspension into each well.

Add 25 μ l of physiological saline containing 1% rabbit serum on a UV type microtitre plate and carry out a negative control test by the same procedure as that for test sample starting from "add 25 μ l of diphtheria antitoxin diagnostic erythrocyte suspension into each well".

Typical result "-" shall be observed in the negative control wells, otherwise the test is invalid and shall be repeated. The appearance of result "++" is served as the reaction end point. The diphtheria antitoxin potency of test sample is calculated by the following formula:

Diphtheria antitoxin potency (HAU/g) of test sample = $(E \times D) / F$

Where: E = Potency of diphtheria antitoxin standard of the highest dilution showing the result "++", HAU/ml;

D = The highest dilution factor of test sample showing the result "++";

F = IgG content in the test sample of human immunoglobulin for intravenous injection, g/ml; or the protein content of test sample of human immunoglobulin, g/ml.

[Note]

(1) The criterion for judgment is as follows:

"-": Erythrocytes are centralized at the bottom of well and appear as a dense small red point with smooth border.

"+": Most of erythrocytes are centralized at the

bottom of well, and only a few of them are scattered around the bottom.

"++": A part of erythrocytes are agglutinated, and a loose small red loop appears at the center of well bottom.

"+++": Most of erythrocytes are agglutinated and distributed evenly, and only a slightly red loop appears at the center of bottom of well.

"++++": All the erythrocytes are agglutinated and distributed evenly.

(2) The wells of microtitre plate shall be kept clean, and the abrasion on their surfaces shall be avoided, otherwise the erythrocytes are not easy to be deposited, and false positive result may appear easily.

X P Test for Fc Function in Human Immunoglobulin

The method is based on the principle that the Fab fragment of specific antibody (immunoglobulin) can bind to the corresponding coated antigen on human erythrocyte and expose the binding site of complement C1q on the Fc fragment, then activate the subsequent various components of complement. Eventually, the membrane of erythrocyte is attacked and disrupted, then hemoglobin is released. The Fc function of the test sample is determined by the calculation of the function index (I_{Fc}) of human immunoglobulin activating complement with the dynamic curve of hemolytic reaction.

Reagent

(1) PBS; Dissolve 1.02 g of anhydrous disodium hydrogen phosphate, 0.34 g of anhydrous sodium dihydrogen phosphate and 8.77 g of sodium chloride in a quantity of water, adjust pH to 7.2 with 1 mol/L sodium hydroxide or hydrochloric acid solution and dilute to 1000 ml with water.

(2) Calcium and magnesium stock solution; Dissolve 1.10 g of calcium chloride and 5.08 g of magnesium chloride in 25 ml of water.

(3) Barbitol-calcium and magnesium stock solution; Dissolve 51.85 g of sodium chloride and 6.37 g of barbitol sodium in 1000 ml of water. Add 3.125 ml of calcium and magnesium stock solution, adjust pH to 7.3 with 1 mol/L hydrochloric acid solution and dilute to 1250 ml with water. Sterilize by filtration and store at 4°C for use.

(4) Bovine albumin-barbitol buffer; Add 0.15 g of bovine serum albumin into 20 ml of barbitol stock solution, dissolve in water and dilute to 100 ml. Prepare just before use.

(5) 1.3 mg/L tannic acid-PBS (pH 7.2) solution
Solution A; Dissolve 1 mg of tannic acid in 10 ml of PBS (pH 7.2).

Solution B; Mix 0.1 ml of solution A with 7.5 ml

of PBS (pH 7.2) thoroughly. Prepare just before use.

(6) 10% chromium chloride solution: Dissolve 5 g of chromium chloride in 50 ml of physiological saline. The solution may be preserved for not more than 6 months at 4°C.

(7) 1% chromium chloride solution: Mix 0.1 ml of 10% chromium chloride solution with 0.9 ml of physiological saline thoroughly. Prepare just before use.

Preparation of sensitized erythrocytes

Solution A: Pool the group O blood, containing anticoagulant, from at least three healthy donors and wash 3 times with PBS. At the last washing, centrifuge the pooled blood at 2000 r/min for 10 minutes to separate erythrocytes. Suspend a quantity of the packed erythrocytes in 1.3 mg/L tannic acid-PBS at a volume ratio of 1 : 40 and gently shake in 37°C water bath for 30 minutes. Wash 3 times with PBS. Then, prepare into a 2.5% erythrocyte suspension with PBS.

Solution B: Mix the diphtheria toxoid or mumps virus, properly diluted with PBS, with 0.25 ml of 1% chromium chloride solution at a volume ratio of 10 : 1 and shake gently in 37°C water bath for 15 minutes.

Mix solutions A and B at a volume ratio of 1 : 4 and shake gently in 37°C water bath for 30 minutes. Centrifuge and discard the supernatant. Wash the sediment (sensitized erythrocytes) 3 times with PBS. Suspend the erythrocytes with bovine albumin-barbital buffer solution and adjust to a suitable concentration at which the absorbance of suspension is 1.0 ± 0.1 at 541 nm.

Preparation of reference solution

Adjust the pH of reference to 6.8-7.0 with 1 mol/L sodium hydroxide solution and dilute the reference to contain 40 mg of IgG per ml with bovine albumin-barbital buffer solution.

Preparation of test sample solution

Adjust the pH of test sample to 6.8-7.0 with 1 mol/L sodium hydroxide solution and dilute the test sample to contain 40 mg of IgG per ml with bovine albumin-barbital buffer solution.

Procedure

To 0.9 ml of test sample solution, add 0.1 ml of sensitized erythrocytes and mix well. Shake gently in 37°C water bath for 30 minutes. Centrifuge and discard the supernatant. Wash the erythrocyte sediment 3 times with 1 ml of bovine albumin-barbital buffer. Discard 800 μ l of supernatant after the last centrifugation, add 600 μ l of bovine albumin-barbital buffer preheated to 37°C and mix well. Two minutes later, add 200 μ l of complement prediluted to 150 CH₅₀ per ml and mix well. Determine the starting absorbance (A_s) at 541 nm by ultraviolet-visible spectrophotometry (Appendix II A) immediately, then determine the absorbance every other minute to obtain a hemolytic reaction dynamic curve with the

absorbance of test sample at 541 nm against the time. Stop the determination when the absorbance is above the turning point of the curve. Repeat the above-mentioned procedure using 0.9 ml of reference and negative control (bovine albumin-barbital buffer) instead of the test sample respectively. Calculate the slopes of curves of reference, test sample and negative control by the following formula (1), and the function index (I_{Fc}) of test sample activating complement by formula (2).

$$S' = S_{\text{exp}} / A_s \quad (1)$$

$$I_{Fc} = [(S'_s - S'_c) / (S'_r - S'_c)] \times 100\% \quad (2)$$

Where: S' = Slope of S_{exp} after calibration by A_s ;

A_s = Starting absorbance of test sample, reference or negative control at 541 nm;

S_{exp} = Maximum slope between three adjacent points on hemolytic reaction dynamic curve of test sample, reference or negative control;

I_{Fc} = Function index of test sample activating complement;

S'_s = Slope of curve for test sample;

S'_r = Slope of curve for reference;

S'_c = Slope of curve for negative control.

X Q Potency Test for Anti-human T Lymphocyte Immunoglobulin (E-rosette Formation-inhibition Test)

The method is based on the principle that anti-human T lymphocyte immunoglobulin may bind the E-receptor of human lymphocyte and inhibit the specific binding of sheep erythrocytes with lymphocyte E-receptor. The potency of anti-human T lymphocyte IgG in test sample is determined according to its inhibiting rate of binding.

Reagent

- (1) Lymphocyte separating solution (Ficoll solution)
- (2) Hank solution
- (3) Hank solution containing 20% fetal calf serum: On the date of test, add the fetal calf serum, inactivated at 56°C for 30 minutes and adsorbed with sheep erythrocytes, into a quantity of sterilized Hank solution to a final concentration of 20%. Adjust pH to 7.2-7.4 with 0.5 mol/L sodium bicarbonate or hydrochloric acid solution.
- (4) 1% sheep erythrocyte suspension: Collect blood from the jugular veins of sheep, add to Alserver solution and store for not more than 2 weeks. Wash a quantity of sheep erythrocytes 3 times with physiological saline and prepare into a 1% sheep erythrocyte suspension with Hank solution containing 20% fetal calf serum just before test.

(5) Lymphocyte suspension: Mix fresh human intravenous blood containing heparin as an anticoagulant with an equal volume of physiological saline thoroughly, and add slowly onto the surface of an equal volume of lymphocyte separating solution. Centrifuge at 2000 r/min for 20 minutes. Suck out the lymphocyte layer and wash with a quantity of physiological saline. Centrifuge at 1200 r/min for 10 minutes. Discard the supernatant, add a quantity of Hank solution containing 20% fetal calf serum into the precipitate and shake well to obtain a lymphocyte stock solution. Make the stock solution 20-fold dilution with 1% acetic blue solution and count the lymphocytes by microscopy. The lymphocyte suspension is prepared by diluting the lymphocyte stock solution to a concentration of 5×10^6 lymphocytes per ml, according to the result of counting, with Hank solution containing 20% fetal calf serum.

Preparation of test sample solution

Dilute the test sample according to its potency into several suitable concentrations with Hank solution containing 20% fetal calf serum.

Procedure

Add 100 μ l of lymphocyte suspension into 100 μ l of test sample solution of each dilution, in duplicate. Mix well and allow the mixture to stand in 37°C water bath for 30 minutes. Add 100 μ l of 1% sheep erythrocyte suspension, mix well and allow the mixture to stand at room temperature for 15 minutes. Centrifuge at 500 r/min for 5 minutes, allow to stand overnight at 2-8°C. Add 100 μ l of 0.2% trypan-blue solution diluted on the date of test, shake well gently and count the formation rate of E-rosette by microscopy. A control test is performed by the above procedure using 100 μ l of Hank solution containing 20% fetal calf serum instead of test sample solution. Calculate the E-rosette inhibition rate. The highest dilution factor of test sample, in which the E-rosette inhibition rate is more than 25%, is served as the E-rosette inhibiting potency.

X R Potency Test for Anti-human T Lymphocyte Immunoglobulin (Lymphocytotoxicity Test)

The method is based on the principle that anti-human T lymphocyte immunoglobulin may bind to human lymphocytes and destroy them in the presence of complement. The potency of anti-human T lymphocyte immunoglobulin in test sample is determined according to the percentage of dead lymphocytes.

Reagent

(1) Lymphocyte separation solution (Ficoll

solution)

(2) Hank solution

(3) Hank solution containing 20% fetal calf serum: Add fetal calf serum, inactivated at 56°C for 30 minutes, to a quantity of sterile Hank solution to prepare a 20% solution on the date of test. Adjust pH to 7.2-7.4 with 0.5 mol/L sodium bicarbonate solution or diluted hydrochloric acid solution.

(4) Lymphocyte suspension: Mix fresh human intravenous blood containing heparin as an anticoagulant thoroughly with an equal volume of physiological saline. Add slowly onto the surface of an equal volume of lymphocyte separating solution and centrifuge at 2000 r/min for 20 minutes. Suck out the lymphocyte layer and wash with a quantity of physiological saline. Centrifuge at 1200 r/min for 10 minutes and discard the supernatant. Add a quantity of Hank solution containing 20% fetal calf serum into the precipitate and shake well to obtain a lymphocyte stock solution. Make the lymphocyte stock solution 20-fold dilution with 1% acetic blue solution and count the lymphocytes by microscopy. Dilute the lymphocyte stock solution with Hank solution containing 20% fetal calf serum into a suspension at a concentration of 5×10^6 lymphocytes per ml according to the result of counting.

(5) Complement: Normal rabbit serum used as complement shall have no significant toxicity to the target cells used in the test. The rabbit serum shall be selected prior to the test by the following method: To 0.05 ml of lymphocyte suspension, add 0.05 ml of 1:5 diluted rabbit serum and allow the mixture to stand at 37°C for one hour. Add 0.05 ml of physiological saline containing 0.5% trypan-blue, incubate at 37°C for 5 minutes and then count lymphocytes by microscopy. Only the rabbit serum, in which the number of dead cells is less than 10%, may be used as complement.

(6) Physiological saline containing 0.5% trypan-blue

(7) 2.5% glutaraldehyde solution (diluted with Hank solution)

Preparation of test sample solution

Dilute the test sample with Hank solution containing 20% fetal calf serum to several suitable concentrations according to its potency.

Preparation of positive control solution

Heat the porcine plasma or rabbit serum, which have been immunized with human T lymphocytes, at 60°C for 10 minutes and make 10-fold dilution with physiological saline.

Preparation of negative control

Heat normal porcine plasma or rabbit serum at 60°C for 10 minutes and make 10-fold dilution with physiological saline.

Procedure

To 0.05 ml of test sample solution, add 0.05 ml

of lymphocyte suspension and allow the mixture to stand at 37°C for one hour. Add 0.05 ml of 1 : 5 diluted rabbit serum, allow to stand at 37°C for 30 minutes. Add 0.05 ml of physiological saline containing 0.5% trypan-blue, allow to stand at 37°C for 5 minutes. Count the lymphocytes immediately by microscopy and calculate the percentage of dead cells. One hundred lymphocytes are counted in general. A positive control test is carried out by the above procedure using 0.05 ml of positive control solution instead of test sample. A negative control test is carried out by the above procedure using 0.05 ml of negative control solution instead of test sample.

Result evaluation

The test is valid if the percentage of dead cells is more than 20% in positive control group and less than 10% in negative control group. The result "+" is served as the end point for judgment. The highest dilution factor of test sample which shows the result of "+" is defined as lymphocytotoxic potency.

[Note]

(1) The result is evaluated by the percentage of

dead cells in test group:

Percentage of dead cells	Result
Less than 10%	(-)
10%-20%	(±)
21%-40%	(+)
41%-60%	(++)
61%-80%	(+++)
Not less than 81%	(++++)

(2) To avoid the experimental error when the test sample is in a large quantity or there is no enough time to observe the result, 0.05 ml of physiological saline containing 0.5% trypan-blue may be added into the test sample after the reaction of antigen, antibody and complement. Allow the mixture to stand at 37°C for 5 minutes and add 0.05 ml of 2.5% glutaraldehyde solution immediately. Then, count by microscopy at an appropriate time. Alternatively, add 0.05 ml of 2.5% glutaraldehyde solution into test sample and allow the mixture to stand at room temperature for 10 minutes at first. Then, add 0.05 ml of physiological saline containing 0.5% trypan-blue and allow to stand at 37°C for 5 minutes. Count by microscopy at an appropriate time.

Appendix XI

XI A Potency Test for Rabies Vaccine for Human Use (NIH Method)

The immunogenicity of rabies vaccine for human use is determined by the changes of antibody levels in mice immunized with test sample.

Reagent

Diluent (PBS): Dilute 75 ml of 0.9% potassium dihydrogen phosphate solution, 425 ml of 2.4% disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) solution and 500 ml of 8.5% sodium chloride solution to 5000 ml with water. Adjust pH to 7.2-8.0.

Preparation of challenging virus strain CVS

Resuspend the seed virus and dilute to a 10^{-2} suspension. Inoculate i.c. 0.03 ml of the suspension into each of not less than eight mice weighing 11-13 g. Undergo the virus in mice for two to three passages. Harvest the brain tissues of the mice with typical rabies signs on the 4th or 5th day after inoculation. Grind the brain tissue to make a 20% suspension with 2% horse or calf serum solution. Centrifuge the suspension at 1000 r/min for 10 minutes and collect the supernatant for sterility test and virus titration using ten mice each weighing 18-20 g. The supernatant qualified in sterility tests shall be used for challenge.

Dilution of reference vaccine

Dilute the reference vaccine with PBS to make a serial dilutions of 1:25, 1:125 and 1:625, etc.

Preparation of test sample solution

Make the test sample 5-fold dilutions of 1:5, 1:25, 1:125, 1:625, etc serially.

Procedure

Inoculate i.p. 0.5 ml of each dilution of test sample and reference vaccine into sixteen mice each weighing 12-14 g, respectively. Inoculate again at an interval of 7 days.

Fourteen days after the first injection, inoculate i.c. each mouse with 0.03 ml of the challenging virus containing 5-100 LD_{50} , which was predetermined. At the same time, dilute the challenging virus into four dilutions (10^0 , 10^{-1} , 10^{-2} and 10^{-3}) for virulence titration, at least eight mice

are required for each dilution. Observe the animals daily for 14 days starting from the date of challenge, and record the death. The mice that die or manifest typical signs of encephalopathy on or after the 5th day following challenge shall be included in the evaluation.

Calculate the ED_{50} values of the test sample and reference vaccine.

The relative potency of vaccine shall be calculated by the following equation:

Potency (IU/ml) of test sample =

$$(T/S) \times (d_T/d_S) \times D$$

Where: T = Reciprocal of ED_{50} of test sample;

S = Reciprocal of ED_{50} of reference vaccine;

d_T = A single human dose of test sample, ml;

d_S = A single human dose of reference vaccine, ml;

D = Potency of reference vaccine, IU/ml

[Note]

(1) The vaccine shall be kept in an ice-bath during animal immunization.

(2) All groups of animals shall be fed under the same condition.

(3) More than 80% of mice inoculated with original challenging virus suspension (dilution 10^0) shall die.

XI B Potency Test for Adsorbed Tetanus Vaccine

The potency of adsorbed tetanus vaccine is calculated by comparing the survival rates of mice (or guinea pigs), which have been immunized with test sample or standard and then challenged with tetanus toxin.

Preparation of standard and test sample solutions

Prepare 3-5 dilutions of the test sample and the standard with physiological saline at a proper ratio separately (the median dilution shall protect about half of animals after challenge).

Procedure

Inoculate s.c. 0.5 ml (or 1 ml for guinea pig) of each dilution of standard and test sample solutions into each of at least fourteen mice weighing 14-16 g (or each of at least ten guinea pigs weighing

250-350 g) respectively. Use ten unimmunized mice (or five unimmunized guinea pigs) as control. Four weeks after immunization, challenge s.c. each of immunized mouse with 0.5 ml of tetanus toxin containing 50 LD₅₀ (or 1.0 ml containing 100 LD₅₀ for guinea pig), and each of the control mice with 0.5 ml of tetanus toxin containing 1 LD₅₀ (or 1.0 ml containing 1 LD₅₀ for guinea pig). Record the death of animal daily for 5 days following challenge. The result shall be calculated by parallel line analysis according to the survival rate on the 5th day after challenge. The 95% confidence interval shall be not greater than 50%-200% of the potency, otherwise the lower 95% confidence limit shall be greater than the required potency specification of the corresponding product.

[Note] The test is valid provided that:

- (1) The lowest dilution of test sample protects more than half of animals;
- (2) The highest dilution of test sample protects less than half of animals;
- (3) The dose-response curves of the test sample and standard do not deviate significantly in parallelism and linearity;
- (4) Animals in control group die partially but not totally.

XI C Potency Test for Adsorbed Diphtheria Vaccine

Method 1 Toxin Challenge Method in Guinea Pig (abitation mthod)

The potency of adsorbed diphtheria vaccine is determined by comparing the survival rates of animals, which have been immunized with test sample and standard and then challenged with diphtheria toxin.

Preparation of standard and test sample solutions

Prepare 3-5 dilutions of the test sample and the standard with physiological saline at an equal proportion separately (the median dilution shall protect about half of the animals after challenge).

Procedure

Inoculate s.c. 1.0 ml of each dilution of standard and test sample solutions into each of at least ten guinea pigs weighing 250-350 g respectively. Use five unimmunized guinea pigs as control.

Four weeks after immunization, challenge s.c. each immunized guinea pig with 1.0 ml of diphtheria toxin containing 100 LD₅₀ and each of the control animals with 1.0 ml of 100-fold diluted toxin mentioned above. Record the death of animals daily for 5 days following challenge. Calculate the potency of test sample by parallel line analysis according to the survival rate on the 5th day after challenge, using the potency of diphtheria toxoid standard as a standard. The potency of test

sample per single human dose shall be not less than 30 IU. If the 95% confidence interval is greater than 50%-200%, the lower 95% confidence limit of the estimate of potency shall be greater than 30 IU per single human dose.

[Note]

The test is valid provided that:

- (1) The lowest dilution of test sample protects more than half of the animals;
- (2) The highest dilution of test sample protects less than half of the animals;
- (3) Animals in control group die partially but not totally;
- (4) The dose-response curves of the test sample and standard do not deviate significantly in parallelism and linearity.

Method 2 Mouse-Vero Cell Antibody Titration Method

The potency of adsorbed diphtheria vaccine is determined by measuring the antitoxin levels in sera of mice immunized with test sample and standard separately, using Vero cell method.

Reagent

- (1) MEM: Just before use, add calf serum, 3% glutamine solution, penicillin and streptomycin into MEM to final concentrations of 10%, 0.03%, 100 IU per ml and 100 µg per ml respectively. Adjust pH to 7.0-7.2 with 7% sodium bicarbonate solution.
- (2) Calcium and magnesium ions-free buffer solution: Dissolve 8.0 g of sodium chloride, 0.2 g of potassium chloride and 1.15 g of disodium hydrogen phosphate in water and dilute to 1000 ml.
- (3) 0.25% trypsin solution: Dissolve 2.5 g of trypsin and 0.2 g of disodium ethylene diamine tetraacetate with calcium and magnesium ions-free buffer solution and dilute to 1000 ml. Adjust pH to 7.0 with 7% sodium bicarbonate solution.

Preparation of Vero cell suspension

Incubate the Vero cell in a 150 cm² flask. Discard the culture medium in the upper layer when the cell monolayer is confluent and about 80%-100% full. Add 10 ml of 0.25% trypsin solution and digest at 37°C for several minutes. Discard the trypsin solution and add 10 ml of medium to disperse the cells. Count the cells and dilute to a concentration of 2.5×10^5 cells per ml with the medium.

Dilution of standard and test sample

Prepare three to five serial 2-fold dilutions of the standard and test sample of adsorbed diphtheria vaccine with physiological saline separately.

Procedure

- (1) Immunization and bleeding

Inoculate s.c. 0.5 ml of each dilution of standard and test sample into each of eight NIH mice weighing 10-14 g respectively. Bleed each mouse 5 weeks after injection, separate the serum, inactivate at 56°C for 30 minutes and store at -20°C.

(2) Positive control serum

Immunize a number of mice with adsorbed diphtheria vaccine and bleed 5 weeks later. Separate the serum, inactivate at 56°C for 30 minutes, dispense it in small tubes, lyophilize and then store at -20°C.

(3) Determination of test dose of toxin

The concentration of toxin used to determine diphtheria antibody titer by Vero cell method is 1/10000 Lcd.

Dilute the toxin 2-fold serially with MEM on a 96-well microtitre plate, 50 µl per well. Add 50 µl of diphtheria antitoxin standard (0.0001 IU) to each well. Lid a cover and allow the plate to stand at room temperature for one hour. Add 50 µl of Vero cell suspension to each well. Lid a cover, seal the plate with a piece of film, incubate at 37°C in a 5% carbon dioxide incubator for 6-7 days and observe the result. The highest dilution that causes the death of cells (red colour) is defined as 1/10000 Lcd.

The amount of 1/10000 Lcd of toxin is equivalent to 1×10^{-4} Lf and is suitable for the test.

(4) Antibody titration

The test is carried out on a 96-well microtitre plate.

① Add 50 µl of medium to each well except A11, A12 and H11, H12. Add 100 µl of medium to wells G11 and G12.

② Add eight serum samples to be tested to wells A1 to H1 respectively, 50 µl per well. Carry out serial 2-fold dilutions horizontally to wells A10 and H10 respectively.

③ Dilute the diphtheria antitoxin standard to a concentration of 0.008 IU per ml. Add 50 µl to each of wells A11, A12, B11 and B12, then carry out serial 2-fold dilutions vertically from wells B11 and B12 to wells D11 and D12 respectively.

④ Add 50 µl of positive control serum to wells H11 and H12 respectively.

⑤ Add 50 µl of toxin (Lcd/10000) to each well except G11 and G12 and mix well. Lid a cover and allow the plate to stand at room temperature for one hour.

⑥ Collect and count the Vero cell suspension, and then dilute to a concentration of 2.5×10^5 cells per ml.

⑦ Allow the microtitre plate to stand at room temperature for one hour. Add 50 µl of cell suspension immediately to each well. Lid a cover, seal the plate with a piece of film and incubate at 37°C in a 5% carbon dioxide incubator for 6 - 7 days.

⑧ Take out the plate and record the result according to the change of colour. Yellow and red colours represent positive and negative results respectively. The results can be examined by microscopy when the change of colour is not clear. If the cell monolayer is intact, the result shall be judged as positive. Otherwise it shall be negative. The final result is expressed as the exponent of 2.

That is, the exponent of highest dilution of serum showing yellow colour is the end point. For example, if the highest dilution is 256 (2^8) folds, the result shall be recorded as 8.

The result shall be calculated by parallel line analysis. The dose-response curves of the test sample and standard shall not deviate significantly in parallelism and linearity. The potency of test sample per single human dose shall be not less than 30 IU. If the 95% confidence interval is greater than 50%-200%, the lower 95% confidence limit shall be greater than 30 IU per single human dose.

[Note]

The requirements for the test are as follows:

(1) All the results of wells E11, E12, F11 and F12, which represent the amount of toxin and the sensitivity of Vero cell, are negative. If they are positive, both the amount of toxin and sensitivity of cells are very low, and the test shall be repeated.

(2) All the results of wells G11 and G12 and positive wells H11 and H12 must be positive. If they are negative, the test shall be repeated.

(3) If the amount of toxin used is correct, all the results of wells A11, A12, B11 and B12 shall be positive, and those of wells C11, C12, D11 and D12 shall be negative. Otherwise, the test shall be repeated.

(4) The cell counting shall be accurate.

XI D Determination of Flocculation Unit of Toxoid

The flocculation unit (Lf) of toxoid is determined by the reaction of toxoid with the corresponding antitoxin in test tubes when their contents, ratio, reaction temperature and reaction time are optimal.

Reagent

Borate buffer solution: Dissolve 0.5 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), 4.5 g of boric acid and 8.5 g of sodium chloride in 1000 ml of water. The pH of the buffer solution is 7.0-7.2.

Preparation of standard solution

Measure the national standard of diphtheria or tetanus flocculating antitoxin accurately and dilute with borate buffer solution to contain 100 Lf per ml.

Preparation of test sample solution

Dilute test sample with borate buffer solution to a suitable limit of flocculation unit.

Procedure

Accurately measure 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml and 0.7 ml of 100 Lf per ml standard flocculating antitoxin (SFA) and add into five flocculating reaction tubes respectively. Then accurately measure 1 ml of test sample solution and add

rapidly into each of the above tubes. Mix well and put the tubes into 45°C water bath. Observe continuously the appearance of flocculating phenomenon in each tube. Record the SFA volume and time (kf) of the tube in which the flocculating phenomenon appears first. Repeat this test with another five flocculating reaction tubes. Accurately measure the same SFA volume as that of the tube in which flocculating phenomenon appears first in the last test, add into one of the five tubes and put this tube in the middle. Put the other four tubes on the two sides of this tube, two for each. To the two tubes at one side, add SFA volumes increasing by 0.05 ml in turn compared with that of the tube in the middle, and, to the two tubes at the other side, add SFA volumes decreasing by 0.05 ml in turn. Add 1 ml of the test sample into each of the five tubes, mix well and put into 45°C water bath. Observe the appearance of flocculating phenomenon. Record again the SFA volume and time (kf) of the tube in which the flocculating phenomenon appears first. Repeat the test using another five tubes. However, the interval between the SFA volumes in the five tubes is 0.02 ml instead. Record again the SFA volume and time (kf) of the tube in which the flocculating phenomenon appears first. Repeat this test 2-3 times. The identical result obtained from two to three tests is defined as the final determination result.

The flocculation unit of test sample shall be calculated by the following equation:

$$\text{Flocculation unit of test sample (Lf/ml)} = \frac{E \times F \times 100}{\dots}$$

Where: E = Volume of 100 Lf/ml SFA in which flocculating phenomenon appears at first, ml;

F = Dilution factor of test sample.

XI E Potency Test for Diphtheria Antitoxin (Rabbit Skin Test)

The potency (IU/ml) of diphtheria antitoxin is calculated by comparing the test sample with standard based on the principle that toxin can be neutralized by antitoxin.

Reagent

Diluent (borate buffer solution): Dissolve 8.5 g of sodium chloride, 4.5 g of boric acid and 0.5 g of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in water, dilute to 1000 ml and filter. After sterilization, the pH of the solution shall be 7.0-7.4.

Preparation of standard solution

The diphtheria antitoxin standard shall be diluted to contain 1/15 IU per ml, that is, each 0.1 ml of the injecting dose contains 1/300 IU after mixing the standard with an equal volume of toxin. The

volume of stock solution of the antitoxin standard in one measurement shall be not less than 0.5 ml.

Preparation of test sample solution

Prepare several dilutions of the test sample to contain about 1/15 IU per ml. The interval of dilutions is about 5%-10%.

Procedure

The toxin is diluted to contain 20 test doses per ml, that is, each 0.1 ml of injecting dose contains one test dose (Lr/300) after mixing the toxin with an equal volume of antitoxin. A quantity of diluted antitoxin standard and different dilutions of test sample solution are measured and transferred to test tubes respectively. Add an equal volume of the diluted toxin to each tube and mix well. Stopper the test tubes, bind at 37°C for one hour, and inject into rabbits immediately.

Healthy rabbits with white skin, each weighing 2-3 kg, are used. Remove hair on the back of the rabbits by an appropriate method one day before the test. If inflammation or a large number of spots are found on the skin, the rabbit shall not be used. Inject each sample solution into two rabbits, four sample dilutions for each rabbit at the most. Inject i. d. 0.1 ml of each dilution into the rabbit at both sides of spine. Control test shall be carried out on at least three different injection sites (front, middle and rear) for each rabbit. Different syringes shall be used for the injection of standard solution and test sample solution.

Result evaluation

The test rabbits shall be observed 48 and 72 hours after injection respectively, and the sizes of reaction areas shall be measured. The final result shall be evaluated by the reactions observed during 48-72 hours. Generally, mild redness at the sites of injection with control occurs during 48-72 hours and their diameters are 10-14 mm. The highest dilution causing the same reaction intensity as that caused by most controls is regarded as the potency of test sample. However, the reaction intensity caused by the test sample shall not exceed that caused by the control.

The test shall be repeated if one of the following cases occurs.

- (1) The reactions of the control do not conform to the criteria mentioned above.
- (2) The dilutions of test sample are too high or too low.
- (3) The reactions are irregular.

[Note]

The toxin is provided by the NCL or prepared by the manufacturer, but only that with a proper toxicity and has been stored for more than 12 months shall be used. The toxin used for testing shall be accurately calibrated for its test dose (Lr/300) with the antitoxin standard distributed by the NCL, and the calibration shall be repeated

once 3 months. The toxin shall be stored at 2-8°C, protected from light, and toluene or other appropriate preservatives shall be added.

XI F Potency Test for Tetanus Antitoxin (Mouse Bioassay)

The potency (IU/ml) of tetanus antitoxin can be calculated by a comparative test on the test sample and antitoxin standard based on the principle that toxin can be neutralized by antitoxin.

Reagent

Borate buffer saline; Dissolve 8.5 g of sodium chloride, 4.5 g of boric acid and 0.5 g of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in water and dilute to 1000 ml, then filter. After sterilization, the pH of the solution shall be 7.0-7.2.

Preparation of standard solution

(1) Dilution of tetanus antitoxin standard; The tetanus antitoxin standard is diluted with borate buffer saline to contain 0.5 IU per ml, that is, each 0.4 ml of injecting dose shall contain 1/10 IU after mixing the standard with an equal volume of toxin. The volume of stock solution of tetanus antitoxin standard in one measurement shall be not less than 0.5 ml.

(2) Dilution of tetanus toxin; The toxin is diluted with borate buffer saline to contain 5 test doses ($L+10$) per ml, that is, each 0.4 ml of injecting dose contains 1 test dose ($L+10$) after mixing the toxin with an equal volume of antitoxin. The test dose ($L+10$) of toxin used shall be accurately calibrated against the antitoxin standard distributed by the NCL and the calibration shall be repeated once 3 months.

Preparation of test sample solution

Prepare several dilutions of the test sample with borate buffer saline to contain about 0.5 IU per ml, that is, each 0.4 ml of injecting dose contains about 1/10 IU after mixing the antitoxin with an equal volume of toxin. The interval of dilutions is about 5 %.

Procedure

A quantity of diluted antitoxin standard and the different dilutions of test sample solutions shall be measured and transferred to test tubes respectively. Add an equal volume of the diluted toxin into each tube and mix well. Stopper the test tubes. Bind at 37°C for one hour and inject into mice immediately.

A portion of 0.4 ml of each mixture is injected s. c. into the abdomen or inguinal part of each mouse weighing 17-19 g. Care shall be taken to avoid overflow of the injecting contents. At least three mice shall be used for each dilution of test sample and control respectively. Different syringes shall

be used for the injection of control and test sample. The same syringe may be used for injecting different dilutions of the same sample in the order from high dilution to low dilution. The syringe shall be washed 2-3 times with the next dilution when changing dilution. The mice shall be observed at least twice a day in the morning and afternoon. Record the morbidity and death of mice for 5 consecutive days.

Result evaluation

All the mice in control group shall die within 72-120 hours.

The highest dilution of the test sample, which causes the death or neurotoxic symptoms of mice in test and control groups at the same time shall be regarded as the potency of test sample.

The test shall be repeated if one of the following cases occurs.

- (1) The dilutions of test sample are too high or too low.
- (2) The mice in control group die within 72 hours or more than 120 hours after injection.
- (3) The death of mice is irregularly, or nonspecific death occurs in more than two mice injected with the same dilution.

[Note]

The dry toxin used shall be weighed accurately and each weighing shall be not less than 10 mg. The toxin after being dissolved shall be used up at one time. The remaining dry toxin shall be stored in a sealed vacuum container with desiccant. The dry toxin may also be made into a liquid form by dissolving with physiological saline and mixing with an equal volume of neutral glycerol (sterilized at 116°C for 10 minutes). Each ml of the liquid toxin shall contain at least 20 test doses. The toxin shall be stored at 2-8°C and protected from light.

XI G Potency Test for Gas-gangrene Antitoxins (Mouse Bioassay)

The potency of gas-gangrene antitoxins is determined by comparing the survival and death of mice injected with different dilutions of test sample and antitoxin standard after combination with the corresponding toxin, based on the principle that toxin can be neutralized by antitoxin.

Reagent

Diluent; Dissolve 8.5 g of sodium chloride, 4.5 g of boric acid and 0.5 g of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in water and dilute to 1000 ml, then filter. After sterilization, the pH of the solution shall be 7.0-7.2.

Preparation of gas-gangrene toxin solution; The toxins shall be provided by the NCL or prepared by

the manufacturer. The test doses of toxins used for testing shall be accurately calibrated with the antitoxin standard distributed by the NCL (see the table below) once 3 months. The toxins shall be diluted with the diluent just before use to make each ml contain 5 test doses (20 test doses for *Cl. oedematiens*).

Preparation of gas-gangrene antitoxin standard solution

Gas-gangrene antitoxin standards (*Cl. perfringens*, *Cl. oedematiens*, *Cl. septicum* and *Cl. histolyticum*) shall be provided by the NCL, stored at 2-8°C and protected from light. The antitoxin standards are diluted with the diluent to make each ml contain the titers as indicated in the table below. The volume of the stock solution of antitoxin standards in one measurement shall be not less than 0.5 ml.

Preparation of test sample solution

Prepare several dilutions of the test sample with the diluent to contain about 5 test doses per ml (20 test doses for *Cl. oedematiens*). The interval of dilutions is about 5%-10%.

Procedure

Measure accurately 0.8 ml, 1.0 ml and 1.2 ml of gas-gangrene antitoxin standard solutions and transfer to a set of test tubes separately. Add 0.7 ml, 0.5 ml and 0.3 ml of the diluent following the order of tubes. Measure accurately 1.0 ml of each dilution of test sample and transfer to a series of test tubes separately. Add 0.5 ml of the diluent to each tube. The diluent may be added before the addition of antitoxins. Add 1.0 ml of gas-gangrene toxin solution (0.5 ml for *Cl. oedematiens*) to each of the above-mentioned tubes and mix well. Stopper the test tubes and bind at 20-25°C for one hour. Inject into mice immediately, each weighing 17-19 g. Each dilution shall be injected into four mice. The dosages and injection routes are indicated in the table below.

Table Parameters for the potency test of gas-gangrene antitoxins

Type of antitoxin	<i>Cl. perfringens</i>	<i>Cl. septicum</i>	<i>Cl. histolyticum</i>	<i>Cl. oedematiens</i>
Test dose of toxin	L+/5	L+	L+/2	L+/50
Antitoxin (IU/ml)	1.0	5.0	2.5	0.2
Dilution				
Toxin testing dose/ml	5	5	5	20
Antitoxin (ml)	1.0	1.0	1.0	1.0
Mixing				
Toxin(ml)	1.0	1.0	1.0	0.5
Diluent (ml)	0.5	0.5	0.5	0.5

Continued

Type of antitoxin	<i>Cl. perfringens</i>	<i>Cl. septicum</i>	<i>Cl. histolyticum</i>	<i>Cl. oedematiens</i>
Dose(ml)	0.5	0.5	0.5	0.2
Antitoxin (IU)	1/5	1	1/2	1/50
Injection				
Test dose of toxin	1	1	1	1
Animal	4	4	4	4
Route	i. v.	i. v.	i. v.	i. m.

Result evaluation

The animals are observed twice a day in the morning and afternoon. Record the morbidity and death of mice for 3 consecutive days. In antitoxin standard group, at least two of the four animals injected with the minimum amount (0.8 ml) of antitoxin shall die within 3 days. Compare the numbers of death in antitoxin standard and test sample groups and calculate the potency of test sample.

The test shall be repeated if one of the following cases occurs.

- (1) All or none of the animals in antitoxin standard group die within 3 days, or less than two of four mice injected with the minimum amount of antitoxins die, or more than two of four mice injected with the maximum amount of antitoxins die.
- (2) All or none of the animals in test sample group die within 3 days.
- (3) The death of animals occurs so irregularly that the result can not be evaluated.
- (4) Nonspecific death occurs in more than two animals injected with the same dilution.

[Note]

(1) The bacterial strains and media used, cultural conditions and drying methods used by the manufacturer in toxin preparation shall be consistent with those for the standards distributed by the NCL.

(2) The dry toxins used for test shall be weighed accurately and each weighing shall be not less than 10 mg. The toxins after being dissolved shall be used up at one time. The remaining dry toxins shall be stored in sealed vacuum containers with desiccant. The dry toxins may also be made into a liquid form by dissolving in physiological saline and mixing with an equal volume of neutral glycerol (sterilized at 116°C for 10 minutes). Each ml of toxin shall contain at least 50 test doses. The toxin shall be stored at 2-8°C and protected from light.

XI H Potency Test for Botulinum Antitoxin (Mouse Bioassay)

The potency of botulinum antitoxins is determined

by comparing the survival and death of mice injected with different dilutions of test sample and antitoxin standard after combination with the corresponding toxins, based on the principle that toxin can be neutralized by antitoxin.

Reagent

Diluent: Dissolve 0.7 g of potassium dihydrogen phosphate, 2.4 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 6.8 g of sodium chloride in water for injection and dilute to 1000 ml. Add 2.0 g of gelatin and filter after dissolution. The pH after sterilization shall be 6.2-6.8.

Preparation of botulinum antitoxin standard solution

Dissolve the botulinum antitoxin with physiological saline, mix with an equal volume of neutral glycerol (sterilized at 116°C for 10 minutes) and dilute to a certain concentration. Store at 2-8°C and protect from light. Dilute the botulinum antitoxin standard solution with diluent to make each ml contain the titers as indicated in the table below just before use. The volume of stock solution of botulinum antitoxin standard in one measurement shall be not less than 0.5 ml.

Preparation of botulinum toxin solution

The botulinum toxins shall be provided by the NCL or prepared by the manufacturer. The test dose of botulinum toxins used for testing shall be accurately calibrated once 3 months with the botulinum antitoxin standard distributed by the NCL as indicated in the table below. The toxin solution shall be diluted to make each ml contain 5 test doses just before use.

Preparation of test sample solution

Prepare several dilutions of the test sample to make each ml contain the titers as indicated in the table below. The interval of dilutions is about 5%-10%.

Procedure

Measure accurately 0.8 ml, 1.0 ml and 1.2 ml of botulinum antitoxin standard solution and transfer to a set of test tubes separately. Add 0.7 ml, 0.5 ml and 0.3 ml of the diluent following the order of tubes. Measure accurately 1.0 ml of each dilution of test sample solution, transfer into a set of test tubes separately and add 0.5 ml of the diluent to each tube. Add 1.0 ml of botulinum toxin solution to each of the above test tubes and mix well. Stopper the test tubes and bind at 37°C for 45 minutes. Inject into mice immediately. Each dilution shall be injected into four mice each weighing 14-16 g. The dosages and routes of injections are indicated in the table below.

Result evaluation

The animals shall be observed twice a day in the morning and afternoon. Record the morbidity and death of animals for 4 consecutive days. Compare the end point of 50% animal protection rate in test sample group with the end point of 50% animal mortality rate in antitoxin standard group and calculate the potency of antitoxin.

Table Parameters for the potency test of botulinum antitoxins

Type of antitoxin		A	B	C	D	E	F
Test dose of toxin		1/5 L+	1/10 L+	L+	L+	1/50 L+	1/20 L+
Dilution	Antitoxin(IU/ml)	1.0	0.5	5.0	5.0	0.1	0.25
	Toxin test dose(ml)	5	5	5	5	5	5
Mixing	Antitoxin(ml)	1.0	1.0	1.0	1.0	1.0	1.0
	Toxin(ml)	1.0	1.0	1.0	1.0	1.0	1.0
	Diluent(ml)	0.5	0.5	0.5	0.5	0.5	0.5
Injection	Dose(ml)	0.5	0.5	0.5	0.5	0.5	0.5
	Antitoxin(IU)	1/5	1/10	1	1	1/50	1/20
	Test doses of toxin	1	1	1	1	1	1
	Animal	4	4	4	4	4	4
	Route	i. p.	i. p.	i. p.	i. p.	i. p.	i. p.

The test shall be repeated if one of the following cases occurs.

(1) All or none of animals in antitoxin standard group die, or the death occurs so irregularly that the end point of 50% mortality rate can not be calculated.

(2) All or none of animals in test group die, or the death occurs so irregularly that the end point of 50% protection rate can not be calculated.

(3) Nonspecific death occurs in more than two animals injected with the same dilution.

[Note]

(1) The bacterial strains and media used, cultural conditions and drying methods used by the manufacturer in toxin preparation shall be consistent with those for the standards distributed by the NCL.

(2) The dry toxins used for test shall be weighed accurately and each weighing shall be not less than 10 mg. The toxins after being dissolved shall be used up at one time. The remaining dry toxins shall be stored in sealed vacuum containers with desiccant. The dry toxins may also be made into a liquid form by dissolving in physiological saline and mixing with an equal volume of neutral glycerol (sterilized at 116°C for 10 minutes). Each ml of toxin shall contain at least 20 test doses. The toxin shall be stored at 2-8°C and protected from light.

XI I Potency Test for Snake Antivenins (Mouse Bioassay)

The potency of snake antivenins is determined by comparing the time and number of death of animals injected with different dilutions of test sample and antivenin standard/reference after combination with the corresponding snake venoms, based on the principle that snake venoms can be neutralized by snake antivenins.

Reagent

Diluent: Dissolve 8.5 g of sodium chloride, 4.5 g of boric acid and 0.5 g of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in water for injection, dilute to 1000 ml and filter. The pH of the solution after sterilization shall be 7.0-7.2.

Preparation of snake antivenin standard solution

Dilute snake antivenin standard/reference to make each ml contain 5 U (*Bungarus multicinctus* or *Agkistrodon halys* antivenin), 5 IU (*Naja naja* (atra) antivenin) or 10 U (*Agkistrodon acutus* antivenin). That is, each 0.4 ml of injecting dose contains 1 U or 2 U (or IU) of antivenin after the standard/reference is mixed with 5 test doses of the corresponding venom.

Preparation of snake venom solution

The test dose of snake venom shall be accurately calibrated by the corresponding snake antivenin standard/reference, which is distributed by the NCL (1 L+ for *Agkistrodon halys*, *Naja naja* (atra) and *Bungarus multicinctus* and 2 L+ for *Agkistrodon acutus*). Dilute the snake venom with a volume of not more than 0.8 ml containing 5 test doses. That is, each 0.4 ml of injecting dose contains one test dose of venom after mixing the snake venom with antivenin and adding diluent to a total volume of 2 ml.

Preparation of test sample solution

Prepare several dilutions of the test sample to make each 1.0 ml contain about 5 U (or IU) of *Agkistrodon halys*, *Naja* (naja) atra or *Bungarus multicinctus* antivenin, or about 10 U of *Agkistrodon acutus* antivenin. The interval of dilutions shall be 5%-10%.

Procedure

Transfer 1.0 ml of each dilution of test sample to a series of small test tubes separately. Use 1 ml and 1.2 ml of snake antivenin standard solutions as controls ① and ② respectively. To each of above antivenin tubes, add 5 test doses of venom solution corresponding to the test sample and make up to 2 ml with the diluent, that is, each 0.4 ml of test sample for injection contains 1 or 2 test doses. Mix well and stopper the tubes. Bind at 37°C for 45 minutes and inject to mice immediately.

Inject i. p. 0.4 ml of each dilution of test sample, control ① and control ② into each of four mice weighing 18-20 g respectively.

Result evaluation

Observe the test mice once a day for 48-72 hours and record the morbidity and death. For the test to be valid, not less than 50% of mice in control group ① shall die, and the mice in control group ② shall die later or die less than those in control group ①, or even no death. The highest dilution of the test sample which cause the death of mice at the same time and in the same number as those in control group ① shall be regarded as the potency

of test sample.

The test shall be repeated if the test animals fail to meet the above-mentioned requirements.

[Note]

(1) The mice shall be injected with an accurate dosage and in a right injecting site. Meanwhile, the overflow of injecting contents shall be avoided.

(2) The dry toxins used for test shall be weighed accurately and each weighing shall be not less than 5 mg. The toxins after being dissolved shall be stored at 2-8°C and used up within 3 days. The remaining dry toxins shall be stored in sealed vacuum containers with desiccant. They may also be prepared into a liquid form and mixed each with an equal volume of neutral glycerol (sterilized at 116°C for 10 minutes). One ml of the mixture shall contain at least 50 test doses. The venoms shall be stored at 2-8°C and protected from light.

XI J Potency Test for Rabies Antiserum

The potency of rabies antiserum is determined by comparing the survival and death of mice at specified time, injected i. c. with different dilutions of test sample and the standard of rabies antiserum after combination with rabies virus suspension, based on the principle that rabies virus can be neutralized by rabies antiserum.

Reagent

(1) Phosphate buffer solution containing 2% calf serum: Dissolve 1.2 g of potassium dihydrogen phosphate and 20.8 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) in water for injection, dilute to 1000 ml and filter. Adjust the pH to 7.6. The pH of the solution after sterilization shall be 7.2-8.0. Add 2 ml of inactivated calf serum to 98 ml of the buffer solution just before use.

(2) Phosphate buffer solution containing 20% calf serum: Dissolve 1.2 g of potassium dihydrogen phosphate and 20.8 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) in water for injection, dilute to 1000 ml and filter. Adjust the pH to 7.6. The pH of the solution after sterilization shall be 7.2-8.0. Add 20 ml of inactivated calf serum into 80 ml of the phosphate buffer solution just before use.

Preparation of virus suspension for neutralization

(1) Preparation of virus suspension

Prepare a 10^{-2} virus suspension of CVS strain (generally freeze-dried) according to the method described in section (2) of preparation of virus suspension for neutralization. Inoculate mice i. c. each weighing 10-12 g with 0.03 ml of the suspension. Prepare a 10^{-2} suspension with the brain of mouse showing signs of illness. Inoculate

mice i. c. with the suspension and undergo for two to three passages in general. The brains of mice showing signs of illness and paralysis on the 5th day shall be ground to prepare a 20 % suspension with skimmed milk. Dispense 0.5 ml of the suspension into each container, seal under vacuum after lyophilization to prepare freeze-dried virus for neutralization and store at -30°C for use. Alternatively, grind the brain tissue of mice showing signs of illness and paralysis on the 5th day to prepare a 10^{-1} suspension with phosphate buffer solution containing 20% calf serum. After centrifugation at 2000 r/min for 20 minutes, collect the supernatant, mix well, dispense the mixture into small tubes and store at -70°C for use.

(2) Preliminary determination of virulence for virus suspension

① Preliminary determination of freeze-dried virus

To the freeze-dried virus containing 20% mouse brain suspension, add 1.0 ml of phosphate buffer solution containing 2% calf serum and mix well. Then, add 4.0 ml of phosphate buffer solution containing 2% calf serum and mix well. After centrifugation at 1500 r/min for 10 minutes, add an equal volume of phosphate buffer solution containing 2% calf serum to the supernatant to prepare a 10^{-2} suspension and mix well. Then, make serial 10-fold dilutions of 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} . Transfer 0.5 ml of above dilutions (from 10^{-2} to 10^{-6}) to five small tubes respectively. Add 0.5 ml of phosphate buffer solution containing 2% calf serum to each tube and incubate in 37°C water bath for one hour. Thirty mice each weighing 10-12 g are divided into five groups and injected i. c. with 0.03 ml of the incubated (at 37°C) virus suspensions of 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} respectively. Each dilution shall be given to six mice. Observe the mice daily for 14 days. The deaths within 4 days after inoculation shall be considered as nonspecific. LD_{50} shall be calculated statistically based on the mice dying from the disease beyond 5 days after inoculation.

② Preliminary determination of virus suspension stored at -70°C

Thaw the frozen virus suspension containing 10 % of mouse brain to obtain a 10^{-1} suspension. Prepare serial 10-fold dilutions from 10^{-2} to 10^{-7} . Transfer 0.5 ml of each of 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} and 10^{-3} dilutions to five small test tubes, separately. Add 0.5 ml of phosphate buffer solution containing 2% calf serum to each tube and incubate in 37°C water bath for one hour. Thirty mice each weighing 10-12 g are divided into five groups and injected i. c. with 0.03 ml of the incubated (at 37°C) virus suspensions of 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} and 10^{-3} respectively. Each dilution shall be given to six mice. Observe the mice daily for 14 days. The deaths within 4 days after inoculation shall be considered as nonspecific.

LD_{50} shall be calculated statistically based on the mice dying from the disease beyond 5 days after inoculation.

(3) Preparation of virus suspension used for neutralization

The virus dilution of 100 LD_{50} estimated in preliminary determination shall be used as the dilution of virus suspension for neutralization test in mice. After incubation in 37°C water bath for one hour, the suspension shall contain 32-320 LD_{50} of virus.

Preparation of rabies antiserum standard solution

Antiserum standard shall be provided by the NCL. The working standard of antiserum used by the manufacturer shall be accredited by the NCL. Prepare the standard solution following the instructions.

Preparation of test sample solution

Make the test sample 2-fold dilutions serially with phosphate buffer solution containing 2 % calf serum. Dilutions from 1 : 800 to 1 : 102400 may be generally adopted. But on the basis of the actual titer of test sample, the lowest dilution factor may be appropriately decreased or increased. If the above eight dilutions are adopted, first add 2.7 ml of diluent to 0.3 ml of test sample to make a dilution of 1 : 10. Then, add 3.5 ml of diluent to 0.5 ml of the 1 : 10 diluted sample to prepare a dilution of 1 : 80 and add 2.7 ml of diluent to 0.3 ml of 1 : 80 diluted sample to prepare test sample solution (1) at a dilution of 1 : 800. After that, test sample solutions (2) to (8), at dilutions of 1 : 1600, 1 : 3200, 1 : 6400, 1 : 12800, 1 : 25600 and 1 : 102400 respectively, shall be prepared by serial 2-fold dilution with the addition of 0.5 ml of dilution solution to 0.5 ml of diluted samples starting from solution (1).

Procedure

Add 0.5 ml of test sample solutions (1) to (8) into eight small test tubes separately. Add 0.5 ml of standard of antiserum of the eight dilutions into another eight small test tubes separately. Add 0.5 ml of virus suspension for neutralization test into each the sixteen tubes, incubate in 37°C water bath for one hour and use for injection in mice. In addition, the mice of the same body weight are used to determine the actual LD_{50} of the virus suspension for neutralization test. The method is as follows: Use virus suspension for neutralization test as the original stock to prepare dilutions of 10^0 , 10^{-1} , 10^{-2} and 10^{-3} . Transfer 0.5 ml of each of the above four dilutions to four small test tubes, add 0.5 ml of phosphate buffer solution containing 2% calf serum into each tube and incubate in 37°C water bath for one hour as the neutralization virus control. Inoculate i. c. 0.03 ml of the four dilutions of neutralized test sample, the standard of antiserum and virus suspension control to each mouse weighing 10-12 g respectively. Each dilution shall be injected into six mice. The

inoculation of test sample and standard shall be carried out in an order from low to high dilutions, and the inoculation of virus control shall be carried out from high to low dilutions.

Observe the mice for 14 days and record the morbidity and death of the mice daily. The deaths within 4 days after inoculation shall be considered as nonspecific.

Potency (IU/ml) of test sample = $(B_1/B_2) \times D$

Where: B_1 = Reciprocal ED_{50} of test sample

B_2 = Reciprocal ED_{50} of standard of antiserum

D = International unit of standard of antiserum, IU/ml

XI K Determination of IgG Content (Ultraviolet-visible Spectrophotometry)

The method is based on the principle that IgG may specifically bind to the corresponding antibody to cause agglutination and form antigen-antibody complex at suitable electrolyte concentration, temperature and pH. The IgG content may be calculated according to the absorbance of test sample.

Reagent

(1) Buffer solution: Dissolve 12.42 g of Tris, 9 g of sodium chloride, 50 g of polyethylene glycol (PEG, 6000), 1 g of bovine serum albumin (BSA) and 1 g of sodium azide (NaN_3) in water. Adjust pH to 7.4 with 1.0 mol/L hydrochloric acid solution and dilute to 1000 ml with water.

(2) Anti-human IgG serum: Reconstitute the freeze-dried anti-human IgG serum following the instructions. According to the labelled potency, dilute a certain amount of anti-human IgG serum with buffer solution to a titer of 1 : 4 (for example, to 2 ml of 1 : 100 anti-human IgG serum, add 48 ml of buffer solution). Mix well and filter with a 0.45 μ m membrane. Store at 4°C.

Preparation of IgG standard solution

Dilute the standard IgG with physiological saline to make a series of dilutions at a concentration range of 0.2-6.0 mg per ml. Five dilutions are required in general.

Preparation of test sample solution

Prepare three dilutions (high, medium and low) of the test sample with physiological saline, in which the IgG content is within the limits of standard curve.

Procedure

To 10 μ l of test sample solution at each dilution, in duplicate, add 1 ml of antibody solution preheated to 37°C and mix well. Warm the mixture in 37°C water bath for one hour. Mix well

and read the absorbance at 340 nm by ultraviolet-visible spectrophotometry (Appendix II A).

Repeat the above-mentioned procedure using 10 μ l of IgG standard solution instead of test sample solution.

Calculate the mean absorbance of each dilution of standard and sample respectively. A linear regression equation is obtained by regressing the logarithm of IgG content of the standard solutions with the logarithm of corresponding absorbance. The correlation coefficient of linear regression equation shall be not less than 0.99. Insert the logarithm of absorbance of test sample solutions into the regression equation and calculate the antilogarithm of corresponding IgG contents. Multiply the result with the dilution factor to obtain the IgG content (g/L) in 1 ml of test sample. The IgG content of the test sample is the mean of the IgG contents obtained (g/L).

[Note]

(1) All the measurement of reaction tubes shall be completed within 10 minutes.

(2) The slit width for ultraviolet-visible spectrophotometry shall be designed as 2 nm.

(3) The IgG concentration of standard solution may be properly adjusted according to the IgG content of test sample.

XI L Test for Neurovirulence in Monkeys

The method is used for the control tests on live attenuated poliomyelitis vaccine.

Healthy monkeys each weighing 1.5 kg or above shall be used. The monkey serum after 1 : 4 dilution shall be shown to contain no neutralizing antibody to virus of the same type. The monkeys shall be selected and quarantined, and shall have not been used for other tests. The quarantine period shall be not less than 6 weeks. There shall be no tuberculosis, B virus infection or other acute infectious diseases. The serum shall be free from antibody to foamy virus. The monkeys with serious purulent foci, neoplasm as well as discernable nephro- and hepatopathy shall not be used for the test. Intraspinal or intracerebral injection method can be adopted.

Intraspinal inoculation

(1) Number of monkeys

Reference preparation shall be established. For the evaluation of vaccines and their respective reference preparations of types I and II, at least eleven valid monkeys for each type shall be included. However, for the evaluation of type III, at least eighteen valid monkeys are required. The monkeys with different body weights and sexes shall be allocated randomly in every group. One

reference preparation can be tested in parallel with more than one lot of vaccine.

A valid monkey is the one of which neuronal lesions specific for poliovirus shall be shown in the central nervous system.

It is permitted to fill the vacancies if the valid monkeys are not enough in the test group, and it shall be necessary to supplement the same number of monkeys to the reference group, and vice versa. If the test needs two working days, the number of monkeys used in both test and reference groups shall be the same on each day. In order to ensure the numbers of valid monkeys in the two groups, it is always to inoculate more monkeys than that required.

Virus titers of test sample and reference preparation

The virus contents of the test sample and the reference preparation shall be adjusted to be as close as possible. Inject 0.1 ml (containing 6.5-7.5 lg CCID₅₀ per ml) interspinally between the first and second vertebrae of each monkey. Only the virus suspension at an appropriate dilution shall be used for injecting the animals.

Procedure

All the inoculated monkeys shall be observed for 17-22 days. Monkeys that survive the first 24 hours but die afterwards shall be autopsied to determine whether poliomyelitis is the cause of death. Those dying due to causes other than poliomyelitis shall be excluded from the evaluation. The test is valid if at least 80% of the monkeys in each group survive the observation period. Animals that become moribund or are severely paralyzed shall be killed for autopsy.

Central nervous system of each monkey shall be subjected to histopathological examination. Sections shall be made at a thickness of 10-15 μ m and stained with galloxyanine. The minimum number of sections examined shall be as follows:

12 sections from the whole of the lumbar enlargement;

10 sections from the whole of the cervical enlargement;

2 sections from the medulla oblongata;

1 section each from the pons and cerebellum;

1 section from the mid-brain, and 1 section each from the left and right of the cerebral cortex and the thalamus.

An evaluation of 4-grade-system of scoring on the severity of the lesions shall be used. A specially designated, experienced person shall examine the sections.

Grade 1: Cellular infiltration only (this is not sufficient to consider the monkey as valid).

Grade 2: Cellular infiltration with a small number of neuronal damage.

Grade 3: Cellular infiltration with extensive neuronal damage.

Grade 4: Massive neuronal damage with or without cellular infiltration.

A monkey having neuronal lesions in the section,

but no needle track, shall be regarded as valid. A monkey having damage due to trauma in the sections, but no specific pathological lesions, shall not be regarded as valid.

The severity is assessed based on the accumulation of total scores of readings of the histological sections of lumbar cord (Lc), cervical cord (Cc) and brain (Br). The Lesion Score (LS) for each valid monkey is calculated as follows:

$$LS = (\Sigma Lcs/h + \Sigma Ccs/h + \Sigma Brs/h)/3$$

Note: s/h—score/hemisectons

Then calculate the mean LS for each group of valid monkeys.

Only when the mean lesion score of the reference preparation situates within the range between the upper limit and lower limit, whether the vaccines being tested are qualified can be evaluated according to error constants, C_1 , C_2 and C_3 values. The criteria for evaluation are as follows.

Compare the mean LS of vaccine tested (\bar{X}_{test}) and that of reference (\bar{X}_{ref}).

Qualified: $\bar{X}_{test} - \bar{X}_{ref} < C_1$

Unqualified: $\bar{X}_{test} - \bar{X}_{ref} > C_2$

Retest I: $C_1 < \bar{X}_{test} - \bar{X}_{ref} < C_2$ (retest can only be conducted once)

Retest II: If in the same test the difference between the mean LS of test group and the mean LS of reference group is less than C_1 , while the highest LS of some individual monkey in test group is equal to or more than 2.5 and, moreover, 2-fold more than the highest LS of an individual monkey in the reference group, the test shall be repeated.

Pass in retest: $(\bar{X}_{(test1+test2)} - \bar{X}_{(ref1+ref2)})/2 < C_3$

Fail in retest: $(\bar{X}_{(test1+test2)} - \bar{X}_{(ref1+ref2)})/2 > C_3$

Intracerebral inoculation

Inoculate 0.5 ml of virus sample (not less than 7.0 Lg CCID₅₀ per ml) into the thalamus region of each hemisphere of individual twenty healthy monkeys after being anesthetized. Ten monkeys receive undiluted virus sample and the other ten receive 1:10 diluted sample. Observe the inoculated monkeys for 21 days. The test is valid if not less than 80% of the animals survive the observation period, and the number of valid monkeys is not less than 16. Otherwise, it is necessary to make up the deficiencies. Those die within 48 hours after inoculation or shown nonspecific paralysis shall be excluded from the evaluation. The CNS tissues of the animals which die during the observation period and of those killed at the end of observation period shall be subjected to histopathologic examination. The criteria of evaluation are as follows.

Qualified:

The virus preparation shall be judged as qualified if the examination result accords with one of followings.

① No histopathologic lesion suggestive to poliomyelitis infection found in CNS;

② Two monkeys with less than or equal to low-grade histopathologic lesions;

③ One monkey with less than or equal to mid-grade histopathologic lesions.

Unqualified;

The virus preparation shall be judged as unqualified if the examination result accords with one of followings.

① One monkey with mid-grade histopathologic lesions and, at the same time, another monkey

with more than or equal to low-grade histopathologic lesions;

② One monkey with severe histopathologic lesions.

Retest;

If the test result of a pooled lot from several sub-lots of vaccine does not comply with the requirements, it is permitted to repeat the test for the sub-lots, separately. The results of retest shall be evaluated in accordance with the criteria mentioned above.

Appendix XII

XII A Sterility Test

Sterility test is applied to the biologics, source and subsidiary materials and other substances, which are required to be sterile. A satisfactory result only indicates that no contaminating microorganism has been found in the sample examined under the conditions of the test.

The test for sterility shall be carried out in the cleanliness of class 100 laminar-airflow cabinet located within the cleanliness of class 10000 clean areas, or in an isolated system. All the operations shall be performed strictly under an aseptic condition to avoid contamination of microorganisms. The standard of cleanliness of laminar-airflow area, working table and laboratory environment shall be validated periodically according to the current national standard in *The Test Method for Suspended Particles, Floating Bacteria and Sedimentary Bacteria in Clean Room (Area) of Pharmaceutical Industry*. The isolated system shall be validated according to relevant requirements and the standard of cleanliness of internal environment shall be validated and shall meet the requirements for sterility test.

The diluent, washing solution, medium and laboratory apparatus shall be sterilized by the procedures qualified in validation, unless otherwise specified.

The sterility tests for all the biologics, unless otherwise specified, shall be performed by the method in this Appendix.

When sterility test is applied to the sample of a new product or the product manufactured by a modified procedure, the method for test shall be validated to confirm that the sample has no anti-microbial activity under the conditions of the test or such activity may be neglected.

Culture medium and method for preparation

The media used shall be suitable for the growth of aerobes, anaerobes or fungi and can be prepared according to the following formula. The dehydrated medium produced according to the formula may also be used provided that they comply with the requirements.

1. Thioglycolate liquid medium (used for aerobes

and anaerobes)

Tryptone (or pancreatic digest of casein, 2000 mg of total nitrogen) 15.0 g

Yeast extract (or 200 ml of yeast dialysate) 5.0 g

Glucose 5.0 g

Sodium chloride 2.5 g

L-cystine (or cysteine hydrochloride) 0.5 g

Sodium thioglycolate (or 0.6 ml of thioglycolic acid) 0.5 g

Agar 0.65-0.75 g

Resazurin 0.001 g

Water 1000 ml

Add above-mentioned components, except glucose and resazurin, into water and dissolve by slightly heating. Adjust the pH to make the solution slightly alkaline. Boil the solution and clarify by filtration. Dissolve glucose and resazurin in the solution and mix well. Adjust pH of the solution so that it is 7.1 ± 0.2 after sterilization.

Before the test sample is inoculated, the depth of oxidizing layer (pink colour) shall not exceed one-third of total depth of the medium. Otherwise the medium shall be heated in a boiled water bath for not more than 20 minutes until the pink colour disappears and cooled down immediately. The medium can only be heated once and shall be protected from contamination.

2. Modified Martin-Lester agar medium (used for fungi)

Peptone 5.0 g

Yeast extract (or 100 ml of yeast dialysate) 2.0 g

Glucose 20.0 g

Dipotassium hydrogen phosphate 1.0 g

Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) 0.5 g

Water 1000 ml

Add the above-mentioned components, except glucose, into water and dissolve by slightly heating. Adjust pH to about 6.8 and boil the solution. Dissolve glucose in the solution, mix well and clarify by filtration. Adjust pH of the solution so that it is 6.4 ± 0.2 after sterilization.

3. Nutrient agar medium (used for aerobes)

Peptone 10.0 g

Beef extract 5.0 g

Sodium chloride 5.0 g

Agar 14.0 g

Water 1000 ml

Add above-mentioned components into water and dissolve by slightly heating. Adjust pH to make

the solution slightly alkaline. Boil the solution and clarify by filtration. Adjust pH of the solution so that it is 7.2 ± 0.2 after sterilization.

The above-mentioned media are usually dispensed into tubes or other suitable containers each at a volume of 10 ml, 40 ml, 100 ml or 200 ml (or other suitable volumes). The height of medium in each container is two-fifth of total height of the container. Sterilize the media by the procedures qualified in validation test, then test for sterility. The media qualified in sterility test shall be stored at 2-25°C, protected from contamination and shall be used within 3 weeks.

Test for sensitivity of media

1. Bacterial seeds (Distributed by the NCL)

Aerobes

β -hemolytic streptococcus [CMCC (B) 32210]

Bacillus brevis 7316

Anaerobes

Clostridium sporogenes [CMCC (B) 64941]

Fungi

Candida albicans [CMCC (F) 98001]

2. Preparation of bacterial suspensions

Inoculate the freshly cultured β -hemolytic streptococcus, *Clostridium sporogenes* and *Bacillus brevis* into the media to be tested respectively and incubate at 30-35°C for a certain time (24-48 hours for β -hemolytic streptococcus and *Clostridium sporogenes*, and 18-20 hours for *Bacillus brevis*). Harvest β -hemolytic streptococcus and *Clostridium sporogenes* respectively to make a homogeneous bacterial suspension with sterile 0.9% sodium chloride solution. Draw the suspension of *Bacillus brevis*, transfer to a sterile tube and dilute with sterile 0.9% sodium chloride solution to make a homogenous bacterial suspension. Inoculate *Candida albicans* onto a potato medium and incubate at 20-25°C for 5-7 days, then wash the lawn down with sterile 0.9% sodium chloride solution and prepare a homogenous bacterial suspension. Dilute the above bacterial suspensions to the same concentration as that of the standard opacity tubes (distributed by the NCL), then make 10-fold dilution serially with 0.1% peptone water.

3. Inoculation of medium

Add 1 ml of *Bacillus brevis* and 1 ml of *Clostridium sporogenes* at each dilution of 10^{-8} — 10^{-6} , 1 ml of β -hemolytic streptococcus at each dilution of 10^{-9} — 10^{-7} and 1 ml of *Candida albicans* at each dilution of 10^{-7} — 10^{-5} into 9 ml of medium to be tested respectively. The medium in the tubes for anaerobes shall be not lower than 7 cm in height. Each dilution shall be inoculated into at least three tubes of the medium to be tested, using the uninoculated medium as a control. Incubate the media inoculated with β -hemolytic streptococcus and *Clostridium sporogenes* at 30-35°C for 3 days, with *Bacillus brevis* at 30-35°C for 5 days, and with *Candida albicans* at 20-25°C for 5 days. Record the result daily.

4. Result evaluation

The highest dilution of the bacteria that can grow in two-thirds of the inoculated tubes of a given medium, is regarded as the sensitivity of the medium. The highest sensitivity that can be reached in two out of the three tests is judged as the standard sensitivity.

Sensitivity of media The sensitivities of media shall be 10^{-8} for β -hemolytic streptococcus, 10^{-7} for *Bacillus brevis* and *Clostridium sporogenes* and 10^{-6} for *Candida albicans*.

5. Precautions

(1) In order to avoid cross contamination, it is forbidden to handle two bacterial strains in the same clean room simultaneously.

(2) Each batch of media for sterility test shall be subject to sensitivity test, and only those qualified can be used.

Application of media

1. Thioglycolate liquid medium shall be used for testing the contaminating anaerobic and aerobic microorganisms.

2. For testing live vaccines, more agars shall be added into the medium to make slant.

Sampling quantity and number of containers for a test

1. Bulk and final bulk

The sampling quantity of bulk and final bulk shall be at least 0.1% but not less than 10 ml. If the bulk or final bulk is dispensed into several containers after sterilization by filtration, the sampling quantity of each container shall be not less than 10 ml. Bulk and final bulk shall be sampled as described above at each opening of the container. The sampling quantity of final bulk of diagnostic reagents for *in vitro* test shall be not less than 3 ml.

2. Final product

Sterility test shall be performed on each sub-lot of final product by sampling at random at the early, middle and late stages during the filling process or in the different layers of freeze-drying chamber. The numbers of containers of final products for delivery and for post-marketing surveillance are as follows.

2.1 Final product for delivery

(1) When the number of containers to be filled is 100 or less, at least 5 containers shall be sampled; for 101-500 containers, not less than 10 containers shall be sampled; for 501 containers or more, not less than 20 containers shall be sampled.

(2) For freeze-dried blood products containing more than 20 ml each bottle, if less than 200 bottles are freeze-dried in the chamber, 2 bottles shall be sampled, and for 200 bottles and more, 4 bottles shall be sampled. If the filling quantity per bottle of the product is within the range of 6-20 ml, number of sampling shall be doubled. If the filling quantity per bottle is 5 ml or less, the sampling shall be conducted as described in (1) of Section 2.1.

2.2 Final product for post-marketing surveillance

(1) Eight containers shall be sampled from each batch of biologics except for blood products.

(2) If the filling quantity per bottle of blood product is less than 50 ml, 6 bottles shall be sampled from each batch; if the filling quantity is 50 ml or more, 2 bottles shall be sampled.

2.3 If the result of sterility test needs to be checked, the number of containers sampled is the same as that of the final product for post-marketing surveillance.

2.4 Sampling quantity of each container

(1) Direct inoculation method The sampling quantity of each container for direct inoculation is described in the following table.

Filling quantity (V/ml) of each container	$V < 0.5$	$0.5 \leq V < 5$	$5 \leq V < 20$	$20 \leq V < 100$
Sampling quantity (ml) of each container	Total volume	0.5	1.0	5.0

(2) Membrane filtration method If the filling quantity per container is 100 ml or less, the total volume shall be sampled; if the filling quantity is more than 100 ml, half of the total volume shall be sampled.

Procedure

Sterility tests may be performed by direct inoculation or membrane filtration method. If the nature of test sample permits, membrane filtration method shall be adopted preferentially, unless otherwise specified.

1. Direct inoculation method

(1) The test sample containing preservative shall be cultured at first for enrichment. According to the requirements for sampling quantity and number of containers for a test of final product, take samples container by container. Mix the test samples from 20 containers and inoculate into suitable media. The number of tubes of media is determined according to the total volume of the mixed test samples to be inoculated. The quantities of inoculum and the medium shall be in a proportion described below; If phenol or trichloromethane is used as a preservative, the proportion of inoculum to medium shall be at least 1 : 20; and, if mercury compounds are used as the preservative, or formaldehyde or antibiotics is contained in the product, the proportion of inoculum to medium shall be at least 1 : 50. According to the above-mentioned proportions, inoculate the mixed test sample first into thioglycolate liquid medium for enrichment and the enriched medium shall be not less than 200 ml. Incubate the inoculated medium at 20-25°C for 3-4 days and then transfer 0.5 ml of the incubated culture into each of the two tubes containing 10 ml of thioglycolate liquid medium, nutrient agar slant and modified Martin agar medium. Incubate one tube of the thioglycolate liquid medium and one

tube of nutrient agar slant at 30-35°C, and the rest of the tubes at 20-25°C. A negative control test is performed in parallel by the same procedure using sterile 0.9% sodium chloride solution instead of the test sample. The whole incubation period of time for the enrichment tubes and the subcultured tubes shall be not less than 14 days.

(2) The test sample free from preservative does not need enrichment. According to the requirements for sampling quantity and number of containers for a test on final product of each batch (or sub-lot), take samples container by container and mix well. Ten containers of sample shall be taken and mixed if the volume per container is 5.0 ml or less, and 7 containers of sample shall be mixed if the volume is more than 5.0 ml. The number of tubes of media is determined according to the total volume of the mixed test samples to be inoculated. Inoculate the mixed test samples into thioglycolate liquid medium, nutrient agar slant and modified Martin agar medium directly. The volume of test sample inoculated shall not exceed 10% of the volume of medium. The number of inoculated tubes of thioglycolate liquid medium, nutrient agar slant and modified Martin agar medium shall be in a proportion of 1 : 1 : 1. Inoculate half of all the inoculated tubes of thioglycolate liquid medium and nutrient agar slant at 30-35°C respectively and the rest of tubes at 20-25°C. The inoculated modified Martin agar medium shall be incubated at 20-25°C. A negative control test is performed in parallel by the same procedures using sterile 0.9% sodium chloride solution instead of the test sample. The duration of incubation shall be not less 14 days.

(3) If the test sample is turbid and/or the result is uncertain, test sample shall be taken according to the quantity specified in above table for sterility test by the enrichment procedure described in Direct Inoculation Method.

(4) For the diagnostic reagents for *in vitro* test, only final bulk needs to be tested for sterility, that is to say, samples shall be taken after sterilization by filtration and before the addition of preservative. Inoculate the test samples directly into media, incubate for 8 days and observe the result. If there is growth of microorganisms, the final bulk shall be sterilized by filtration and tested for sterility again. If there is still growth of microorganisms in the retest, the final bulk shall be discarded. If the final bulk is sterilized by filtration after the addition of preservative, the sample shall be taken for sterility test by the enrichment procedure in direct inoculation method. However, the whole incubation period of time for enrichment tubes and subcultured tubes shall be not less than 8 days.

2. Membrane filtration method

A completely closed membrane filter shall be used. The pore size of membrane is not more than 0.45 μm . The diameter of membrane is about 47 mm. A given number of test samples shall be taken as

required and added aseptically into a membrane filter immediately for filtration under increased or reduced pressure. The test sample less than 10 ml shall be diluted with 100 ml of sterile 0.9% sodium chloride or other suitable solvents before filtration. For the test sample containing mercury compound as the preservative, flush the filter membrane after filtration for 3 times with sterile 0.9% sodium chloride solution or other appropriate sterile solvent, 100 ml for each time. After filtration, add 100 ml of thioglycolate liquid medium to each of two filters, and 100 ml of modified Martin medium to the other filter. Incubate one filter added with thioglycolate liquid medium at 30-35°C, and the other two at 20-25°C, for not less than 14 days. A negative control test is performed in parallel by the same procedure using sterile 0.9% sodium chloride solution instead of the test sample.

3. Result evaluation

If both thioglycolate liquid medium and modified Martin medium after incubation are clear or turbid but being proved free from microorganism, and no growth of microorganism is observed on the nutrient agar slant, the test sample passes sterility test. If there is growth of microorganism in any tube of the three media, and the microorganisms are proved originating from test sample, the sample fails to pass sterility test. The test is invalid if one of the following situations occurs;

- (1) The microbiological monitoring data show that the facilities related to sterility test fail to meet the requirements;
- (2) The review on the process of sterility test shows that the procedures are incorrect.
- (3) There is growth of microorganism in negative control tube.
- (4) The microorganisms growing in the tubes containing test sample are identified and confirmed that the contamination is due to unsuitable materials and /or aseptic operations used during sterility test.

If the test is confirmed as invalid, it shall be repeated with the same amount of the test sample by the same procedure as those for original test. If there is no growth of microorganism in the repeated test, the sample is judged as qualified; if there is growth of microorganism, the sample is judged as unqualified.

XII B Test for Mycoplasma

Where the test for mycoplasmas is carried out on master cell bank, working cell bank, virus seed lot, control cells or cells for therapeutic use, both the cultivation method and the indicator cell culture method (DNA staining method) are used. Where the test for mycoplasmas is carried out on

virus harvest or bulk vaccine, the cultivation method is used. The indicator cell culture method may be used for screening of media, if necessary. Other methods accepted by the NCL may also be used.

Method 1 Cultivation Method

Recommended culture media and formulas

(1) Mycoplasma broth medium	
Pig gastric digest	500 ml
Beef extract (1 : 2)	500 ml
Yeast extracts	5.0 g
Sodium chloride	2.5 g
Glucose	5.0 g
Phenol red	0.02 g
pH 7.6 ± 0.2. Sterilize at 121°C for 15 minutes.	

(2) Mycoplasma arginine broth medium	
Pig gastric digest	500 ml
Beef extract (1 : 2)	500 ml
Yeast extracts	5.0 g
Sodium chloride	2.5 g
Glucose	1.0 g
L-arginine	2.0 g
Phenol red	0.02 g
pH 7.1 ± 0.2. Sterilize at 121°C for 15 minutes.	

(3) Mycoplasma semifluid medium

Prepare the medium according to the formula (1). Add 2.5-3.0 g of agar but no phenol red is added.

(4) Mycoplasma agar medium

Prepare the medium according to the formula (1). Add 13.0-15.0 g of agar but no phenol red is added.

Sensitivity test on culture media (Color change unit method)

(1) Strain

Mycoplasma pneumoniae (Strain ATCC 15531) and *Mycoplasma orale* (Strain ATCC 23714) are distributed by the NCL.

(2) Procedure: Inoculate the strain in a suitable mycoplasma medium and incubate at 36 ± 1°C till the colour of the medium changes. After two blind passages, inoculate the culture into the medium to be tested and make a serial 10-fold dilution. For *Mycoplasma pneumoniae*, dilute to 10⁻⁷-10⁻⁹ and then inoculate into mycoplasma broth medium; for *Mycoplasma orale*, dilute to 10⁻³-10⁻⁵ and then inoculate into *mycoplasma arginine* broth medium, three tubes of medium for each dilution. Incubate at 36 ± 1°C for 7-14 days and observe the result of colour changing.

(3) Result evaluation: The highest dilution, which makes a colour change in more than two-thirds of all the inoculated medium tubes, is regarded as the sensitivity of the culture medium. Sensitivity of liquid medium: 10⁻⁸ for *Mycoplasma pneumoniae* (Strain ATCC 15531) and 10⁻⁴ for *Mycoplasma orale* (Strain ATCC 23714).

Procedure

(1) Test sample may be preserved at 2-8°C if the test for mycoplasma is carried out within 24 hours after filling; otherwise it shall be preserved below

—20°C.

(2) Semifluid medium and broth medium (or mycoplasma agar medium) shall be used for the test. Before use, boil the semifluid medium (or agar medium) for 10-15 minutes and cool down to about 56°C. Add inactivated calf serum (The volume ratio of medium to serum is 8 : 2) and a quantity of penicillin, wherever necessary, and make it even by shaking. For broth medium the same components as mentioned above shall be added but boiling is not needed.

Inoculate 0.5-1.0 ml of test sample into each of four tubes containing 10 ml of semifluid medium (which have been cooled to $36\pm1^\circ\text{C}$) and into each of four tubes containing 10 ml of broth medium, and incubate at 35-37°C for 21 days. On the 7th day after inoculation, make a subculture of each inoculated medium by inoculating two fresh tubes of semifluid and broth media and incubate at 35-37°C for 21 days, and the inoculated media, during the incubation, shall be observed every other 3 days.

(3) Result evaluation: The product to be tested passes the test if growth of mycoplasmas has not occurred in any of the inoculated media at the end of incubation. If there is suspected growth of mycoplasmas, the test may be repeated once using twice the amounts of test sample. The product complies with the test if growth of mycoplasma does not occur.

[Note]

Quality control department shall examine the sensitivity of mycoplasma culture media in coordination with medium preparation department periodically.

Method 2 Indicator Cell Culture Method (DNA staining method)

Inoculate indicator cell cultures (with Vero cells free from contamination or other cells accepted by the NCL) with the test sample and then stain with a specific fluorescent dye. If the test sample has been contaminated with mycoplasmas, the DNA of mycoplasmas attached on the cell surface shall be stained with the fluorescent dye that binds specifically to the DNA of mycoplasma, which can be easily recognized by using fluorescent microscopy.

Reagent

(1) Concentrated solution of bis-benzimide fluorescent dye (Hoechst 33258)

Dissolve 5 mg of bis-benzimide fluorescent dye in 100 ml of Hank balance solution without phenol red or sodium bicarbonate. Stir with a magnetic stirrer at room temperature for 30-40 minutes to make it dissolved completely. Store at -20°C , protected from light.

(2) Bis-benzimide fluorescent dye working solution

Dilute 1 ml of concentrated solution of bis-benzimide fluorescent dye with 100 ml of Hank balance solution without phenol red or sodium bicarbonate. Mix well.

(3) Fixing solution

Mix one volume of acetic acid with three volumes of methanol.

(4) Sealing solution

Mix 22.2 ml of 0.1 mol/L citric acid solution and 27.8 ml of 0.2 mol/L disodium hydrogen phosphate solution with 50.0 ml of glycerin. Adjust pH to 5.5.

Culture media and indicator cells

(1) DMEM complete medium

(2) DMEM medium without antibiotic.

(3) Indicator cells (Vero cell or other cell line which has proved free from mycoplasma contamination)

Digest the incubated Vero cells and prepare a cell suspension at a concentration of 10^5 cells per ml and inoculate 0.5 ml into each well of a 6-well culture plate or other containers. To each well, add 3 ml of antibiotic-free culture medium. Incubate in a 5% carbon dioxide incubator at $36\pm1^\circ\text{C}$ overnight for use.

Treatment of test sample

(1) Cell cultures

Make not fewer than one subculture of test sample in antibiotic-free medium. Collect the supernatant of the culture in a three-day-unchanged medium when a confluent cell sheet is formed.

(2) Viral suspensions

If for viral suspensions the interpretation of results is affected by marked cytopathic effects, the virus may be neutralized using a specific antiserum that has no inhibitory effects on mycoplasmas or a cell culture substrate that does not allow growth of the virus may be used.

(3) Other test samples

The indicator cells used for test shall be those that their growths are not effected by the test sample.

Procedure

Add 2 ml of test sample (supernatant of cell culture) or at least 1 ml of viral suspension or other test sample into the prepared indicator cell culture plate and incubate in a 5% carbon dioxide incubator at $36\pm1^\circ\text{C}$ for 3-5 days. Make not fewer than one subculture of the indicator cell culture and grow the last subculture in a 6-well culture plate with glass covers for 3-5 days. Draw out the culture fluid from the wells, add 5 ml of fixing solution, allow to stand for 5 minutes. Draw out the fixing solution, add another 5 ml of fixing solution and fix for 10 minutes. Draw out the fixing solution again and dry the glass covers in the air. Add 5 ml of bisbenzimidazole fluorescent dye working solution (or other DNA dyes) and put on covers. Allow the plate to stand at room temperature for 30 minutes. Draw out the dye solution and wash each well 3 times with water, 5 ml for each time. Draw out the water and dry glass covers in the air. Add one drop of sealing solution on a clean slide and put the glass cover up side down on the sealing solution to make sealed

slides. Observe with a fluorescent microscope.

Carry out the negative control test with the same procedure by using 2 ml of antibiotic-free culture medium.

Carry out the positive control test with the same procedure by using 2 ml of standard strain known as positive.

Result evaluation

(1) Negative control

Extranuclear fluorescence of the indicator cells shall be examined which shall be greenish-yellow fluorescent light.

(2) Positive control

Besides the cells, fluorescent particulates with irregular pattern of fluorescence on the cell surface can be observed under fluorescent microscope.

The test is valid only when the validity tests of both negative and positive controls proved qualified.

If the test result is negative, the product complies with the test. If the test result is positive or suspected, the test shall be repeated. If the result is positive again, the product fails to pass the test.

XII C Test for Adventitious Viruses

During the seed selection and production process of viral preparations, animal or cell substrates are usually used, so the preparations may be potentially contaminated with adventitious agents, especially adventitious viruses. Tests for adventitious agents on virus strain and cells are necessary to ensure the quality of the preparations.

Tests for adventitious agents in virus seed lots

A sufficient quantity of samples shall be taken from the master or working seed lots for testing adventitious agents. Before the test, neutralize the viruses with specific antibodies from non-human and non-simian origins, apart from an exceptional case. The immunizing agents used to prepare antiserum (or monoclonal antibody) shall be produced in cell culture (or animal) from a species different from that used for the production of the vaccine and free from adventitious agents. If the viruses have been propagated in avian tissues or cells, the antibodies must be of non-avian origin. If chick embryo is used for the test, it shall be obtained from a flock free from specific pathogens.

1. Test in animals

(1) Test in adult mice

Inoculate i. c. each of at least ten adult mice, weighing 15-20 g, with 0.03 ml and inoculate i. p. with 0.5 ml of virus suspension after neutralization with antiserum. Observe the mice for at least 21 days. The test is valid if at least 80% of the inoculated mice survive the observation period.

Carry out an autopsy of all mice that die after the first 24 hours of the test or that show signs of illness, and examine for the pathological evidence of viral infection by direct macroscopical observation. The suspensions prepared with the tissues showing pathological changes are inoculated i. c. and i. p. into at least five additional mice of 15-20 g which are observed for 21 days. The virus seed lot complies with the test if no mouse shows evidence of virus infection.

(2) Test in suckling mice

Inoculate i. c. each of at least ten suckling mice, less than 24 hours old, with 0.01 ml and inoculate i. p. with at least 0.1 ml of virus suspension after neutralization with antiserum. Observe daily for at least 14 days. The test is valid if at least 80% of the inoculated mice survive the observation period. Carry out an autopsy of all mice that die after the first 24 hours of the test or that show signs of illness and examine for the pathological evidence of viral infection by direct macroscopical observation. The suspensions prepared with the tissues showing pathological changes as well as with the brain and spleen are inoculated i. c. and i. p. into at least five additional suckling mice of less than 24 hours old which shall be observed for 14 days. The virus seed lot complies with the test if no suckling mouse shows evidence of viral infection.

2. Cell culture method

(1) Test for non-haemadsorbing viruses

Inoculate the virus suspension after neutralization with antiserum into the cell cultures of human, simian origins and of the same cell as that used for the production of vaccine, respectively. If the virus is grown on human diploid cells, the neutralized virus suspension shall be tested on a separate cultures of the diploid cells. At least six bottles of each kind of cell cultures shall be inoculated, and the volume of virus suspension inoculated into each bottle shall be not less than 25% of the total volume of culture medium. Incubate at $36 \pm 1^\circ\text{C}$ and observe for 14 days. Change the cell culture medium in case of necessity. It complies with the requirements if no CPE appears.

(2) Test for haemadsorbing viruses

Take two bottles of each of above-mentioned cell cultures inoculated with virus on days 6-8 and 14 respectively and test for the presence of haemadsorbing viruses. Add the mixed suspension of 0.2%-0.5% chicken and guinea pig erythrocytes onto the surface of the cell cultures, incubate at $2-8^\circ\text{C}$ for 30 minutes and $20-25^\circ\text{C}$ for another 30 minutes, and examined microscopically. Both the results shall be negative.

3. Test in chick embryo

The virus seed propagated in avian tissues or cells shall be tested for the contamination of avian viruses using chick embryos. Inoculate the virus suspension after neutralization with antiserum into the allantoides of a group of at least five SPF chick

embryos of 9-11 days old with 0.5 ml for each, and into the yolk sacs of a second group of at least five chick embryos of 5-7 days old with 0.5 ml for each. Incubate the chick embryos for 7 days. Collect the allantoic fluid and test for the presence of haemadsorbing viruses with a mixture of chick and guinea pig erythrocytes, and the result shall be negative. The test is valid if at least 80% of the inoculated chick embryos survive for 7 days.

Tests for adventitious agents in cells used for production

1. Test for non-haemadsorbing viruses

(1) Direct observation on cells

A portion of 5% (or not less than 500 ml) of cell suspension of each batch of cells used for production shall remain uninoculated as a control, and cultured in the same cell maintenance medium under the same conditions as those for production. Observe the cell cultures for 14 days by microscopic examination for CPE. It complies with the requirements if no CPE appears. The test is valid if at least 80% of the cell cultures survive the observation period.

(2) Test in cell cultures

Pool the culture supernatant at the end of above-mentioned observation period. Examine for the presence of adventitious agents by inoculation of the cell cultures of human and simian origins. If the vaccine virus is grown in a cell system other than human or simian, the cells of that species but from a separate batch are also inoculated. The amount inoculated shall be not less than the 25% of total amount of the pooled supernatant. For each kind of cell, at least 5 ml of the pooled supernatant shall be tested. Incubate the cultures inoculated with the pooled supernatant under the same condition as that for production. If no CPE appears, the result is negative and complies with the requirements.

2. Test for haemadsorbing viruses

At least 25% of cell cultures used for the above direct observation and cell culture test shall be tested for the presence of haemadsorbing viruses at the end of observation period by the same method as that for virus seed lot.

XII D Pyrogen Test

Pyrogen test is performed by injecting i. v. a given dosage of the test sample into rabbits. Observe the rise of body temperature for a specified period of time to judge whether the limit of pyrogen in the test sample can meet the requirements.

Rabbits used for the test

Healthy male or healthy and non-pregnant female rabbits each weighing 1.7-3.0 kg shall be used. Feed the rabbits with the same forage 7 days before

measuring the body temperature prior to the test. During this period, no abnormal manifestations, such as loss of body weight, problems in spiritual status, appetite or excretions, shall occur. The rabbits, which have not been previously used for pyrogen test shall be selected by screening. Measure the body temperature of each rabbit 3-7 days prior to the test for 8 times at intervals of 30 minutes under the same condition as that for pyrogen test, but no test sample is injected. Only when all the body temperatures in eight measurements are at a range of 38.0-39.6°C, and the difference between the highest and the lowest body temperatures is not more than 0.4°C, the rabbit may be used for pyrogen test. After pyrogen test in which the test sample has shown a satisfactory result, the rabbits used can be reused after rest for at least 48 hours. The rabbits may be reused only once within 5 days for the pyrogen test of blood products, antitoxins or other samples containing the same anaphylactogen. However, if the result of pyrogen test is unsatisfactory, all the rabbits shall not be used again for the test.

Preparation prior to test

The rabbits shall be kept in a place where the ambient temperature shall be as stable as possible 1-2 days prior to the pyrogen test. The temperature difference between the laboratory and the animal house shall be not more than 5°C. The temperature of the laboratory shall be 17-25°C and the temperature change shall be not more than 3°C during the period of test. The laboratory shall be kept quiet and protected from strong light, noise and disturbance of the animals. Withhold food from the rabbits at least one hour prior to the test, and put the rabbits into suitable unit until the test is completed. The accuracy of device used for measuring the body temperature of rabbits shall be within the range of $\pm 0.1^\circ\text{C}$. Insert the thermometer or probe into the rectum of the rabbit to a depth of about 6 cm for at least one and a half minutes. The depth and time duration of insertion is consistent for any rabbit in any test. Measure the body temperature of each rabbit twice in general at an interval of 30 minutes. The difference between the two measurements shall not exceed 0.2°C. The mean of the two measurements is regarded as the normal body temperature of the rabbit. All the normal body temperatures of the rabbits used on the date of test shall be within the range of 38.0-39.6°C, and the difference between normal body temperatures of rabbits of the same group shall be not more than 1°C.

Syringes, needles and other apparatus contacting directly the test sample shall be heated at 250°C for 30 minutes or sterilized by other suitable methods to remove pyrogen.

Procedure

The test sample or the pyrogen-free diluent shall be preheated to 38°C before injection. The injecting dose of test sample shall be determined

according to the requirements of the individual product. However, the injecting dose shall be not less than 0.5 ml and not more than 10 ml per kg of rabbit body weight.

Within 15 minutes after measuring the normal body temperatures of three rabbits, inject slowly the test sample preheated to 38°C at the prescribed dose into the ear vein of each rabbit. Measure the body temperature of each rabbit for 6 times at intervals of 30 minutes. The difference between the highest body temperature among the six measurements and the normal body temperature is regarded as the body temperature rise of that rabbit. If one of the three rabbits shows a temperature rise of 0.6°C or more, or the temperature rise of each of the three rabbits is less than 0.6°C, but the sum of temperature rises of the three rabbits reaches 1.4°C or more, the test shall be repeated using another five rabbits with the same method as mentioned above.

Result evaluation

The test sample passes the pyrogen test if one of the following situations occurs:

- (1) All the temperature rises of three rabbits used for the test are less than 0.6°C, and the sum of temperature rises of the three rabbits is less than 1.4°C;
- (2) Only one of the five rabbits used for repeated test shows a temperature rise of 0.6°C or more, and the sum of temperature rises of the eight rabbits used for the test and repeated test is not more than 3.5°C.

The sample fails to pass the pyrogen test if one of the following situations occurs:

- (1) More than one of the three rabbits used for the test show temperature rises of 0.6°C or more;
- (2) More than one of the five rabbits used for repeated test show the temperature rises of 0.6°C or more;
- (3) The sum of temperature rises of the eight rabbits used for test and repeated test is more than 3.5°C.

When the value of temperature rise of the rabbit is negative, it shall be regarded as 0°C.

XII E Test for Bacterial Endotoxin

The test for bacterial endotoxins is used to detect or quantify endotoxins of Gram-negative bacterial origin using TAL reagent. It is used to determine the limit concentration of bacterial endotoxin in a preparation being examined.

Two methods are used for this test: the gel-clot method and the photometric method. The latter includes a turbidimetric method and a chromogenic method. Proceed by any one of these two methods. In the event of doubt or dispute, the final decision is made based on the gel-clot

method, unless otherwise indicated in the monograph.

The quantities of endotoxin are expressed in Endotoxin Units (EU).

The National Standard for Endotoxin (NSE) is prepared and purified from *Escherichia coli*. It is used only for calibration of the working standard for endotoxin (WSE) and for calibration and verifying the sensitivity of TAL reagents.

The working standard for endotoxin (WSE) of which the potency has been standardized against NSE in collaboration assay, is used for bacterial endotoxin test as positive control, for interference test and for sensitivity test of TAL reagent.

The water used in the gel-clot test for bacterial endotoxins is a sterile water for injection, of which quantities of bacterial endotoxins are less than 0.015 EU per ml. The quantities of bacterial endotoxins of water used in the photometric test are less than 0.005 EU per ml.

Depyrogenate all glassware and other heat-stable apparatus used in the test by heating in a hot-air oven at 250°C for at least 60 minutes (or by any other validated suitable method) to eliminate extraneous endotoxin that may present. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipettors, use apparatus shown to be free of detectable endotoxin and of interfering effects for the test. Avoid microbial contamination during the test.

Preparation of the test solutions Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for bacterial endotoxins test (water for BET). Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the TAL reagents and test solution falls within the pH range specified by the TAL reagents manufacturer. This usually applies to a product with a pH in the range of 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer, as recommended by the TAL reagents manufacturer. Acids and bases may be prepared from concentrates or solids with water for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

Establishment of endotoxin limits The endotoxin limit (*L*) for drugs or biological products is usually defined as follows:

$$L = K/M$$

Where *L* is the endotoxin limit for active substances administered parenterally, which is specified in units such as EU/ml, EU/mg, or EU/Unit of biological activity;

K is the threshold human pyrogenic dose of endotoxin per kg of body weight in a

single hour period, which is expressed as EU/(kg · h). For injections, $K=5$ EU/(kg · h); for injections of radio-pharmaceuticals, $K=2.5$ EU/(kg · h); and for intrathecal injections, $K = 0.2$ EU/(kg · h);

M is equal to the maximum recommended human dose of product per kg of body weight in a single hour period, which is specified in units such as ml/(kg · h), mg/(kg · h), or U/(kg · h). Here the human average body weight is 60 kg; the injection period is calculated as 1 hour when the injection is completed within 1 hour.

The endotoxin limit calculated by human dose may be adjusted according to the situation of manufacture and clinical use if necessary. In that case, appropriate reasons for adjustment have to be submitted.

Determination of the Maximum Valid Dilution (MVD) The Maximum Valid Dilution (MVD) is the maximum allowable dilution of the substance being examined at which the endotoxin limit can be determined. Determine the MVD using the following formulae:

$$MVD = cL/\lambda$$

Where L is the endotoxin limit of the substance being examined

c is the concentration of the substance being examined, when L is expressed as EU/ml, c is 1.0 ml/ml; when L is expressed as EU/mg or EU/U, the unit of C is mg/ml or U/ml. Minimum valid dilution concentration, $c = \lambda/L$, may be calculated for drug substance or sterilized powders for injection when MVD is 1.

λ is the labelled sensitivity of TAL reagent in the gel-clot method (EU/ml) or the lowest point used in the standard curve of the turbidimetric or chromogenic method.

Gel-clot method (Method 1)

The gel-clot method detects or qualifies endotoxins based on clotting of the TAL reagent in the presence of endotoxins.

Test for confirmation of labelled TAL reagent sensitivity The labelled sensitivity of TAL reagent (EU/ml) is defined as the lowest concentration of endotoxin that is required to cause the TAL reagent to clot under the conditions specified in the following procedure. The test for confirmation of the labelled TAL reagent sensitivity is to be carried out when each new batch of TAL reagent is used or when there is any change in the experimental conditions which may affect the outcome of the test.

Dissolve NSE or CSE in water for BET according to the labelled sensitivity of TAL reagent (λ), mix for 15 minutes using a vortex mixer. Prepare four replicate series of two-fold dilutions of NSE or CSE using water for BET to produce 4 dilutions with concentration of 2.0λ , 1.0λ , 0.5λ , 0.25λ , mix each dilution for 30 seconds using a mixer. Use 18 tubes of 10 mm × 75 mm in size containing of 0.1 ml of TAL reagent or use 18 original ampoules of 0.1 ml of TAL reagent. Add 0.1 ml of each of 16 endotoxin standard solutions (four standard concentrations in quadruplicate) to each of 16 tubes, and 0.1 ml of water for BET to each of 2 tubes as negative control. Mix gently after each addition, cover the tubes tightly and incubate the tubes vertically at $37 \pm 1^\circ\text{C}$ for 60 ± 2 minutes. Take each tube in turn directly from the incubator and invert it through about 180° in one smooth motion to test the integrity of the gel. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed. Handle the tubes with care to avoid vibration, or false negative may result.

The test is not valid unless all the four tubes of highest concentration (2.0λ) give positive results, all the four tubes of lowest concentration (0.25λ) give negative results and the two tubes of negative control give negative results. Calculate the geometric mean endpoint concentration, i. e. the measured sensitivity of the TAL reagent (λ_c), using the following expression:

$$\lambda_c = \lg^{-1} (\sum X/4)$$

Where X is the log endpoint concentration which is the last positive result in a series of decreasing concentrations of endotoxin.

If this λ_c is not less than 0.5λ and not more than 2λ , the labelled sensitivity (λ) is confirmed and is used in tests performed with this TAL reagent.

Test for interfering factors Prepare solutions A, B, C and D as shown in Table 1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under the *Test for confirmation of labelled TAL reagent sensitivity*.

The test is not valid unless all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled TAL reagent sensitivity. The geometric mean endpoint concentrations of solutions B (E_s) and C (E_t) are determined using the following formulas.

$$E_s = \lg^{-1} (\sum X_s/4)$$

$$E_t = \lg^{-1} (\sum X_t/4)$$

Where X_s is the log endpoint concentration of the solution C. X_t is log endpoint concentration of the solution B.

If both E_s and E_t are not less than 0.5λ and not more than 2λ , the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the solution interferes

with the test. If the preparation being examined interferes with the test at a dilution less than the MVD, repeat the test for interfering factors using a greater dilution, not exceeding the MVD. The

use of a more sensitive TAL reagent permits a greater dilution of the preparation being examined and this may contribute to the elimination of interference.

Table 1 Preparation of solutions in the test for interfering factors by using gel-clot method

Solution	Endotoxin Concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	—	—	—	2
B	2 λ /Test solution	Test solution	1	2 λ	4
			2	1 λ	4
			4	0.5 λ	4
			8	0.25 λ	4
C	2 λ /Water for BET	Water for BET	1	2 λ	4
			2	1 λ	4
			4	0.5 λ	4
			8	0.25 λ	4
D	None/Water for BET	—	—	—	2

Solution A: solution of the preparation being examined that is free of detectable endotoxins.

Solution B: test for interference.

Solution C: control of the labelled TAL reagent sensitivity.

Solution D: negative control (water for BET).

Interference may be overcome by suitable treatment, such as filtration, neutralization, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

When establish a method of bacterial endotoxin test for a new drug, the test for interfering factors should be carried out.

When the source of the TAL reagent, or the

formula or the manufacture process of the substance being examined are changed, or there is any change in the experimental conditions which may affect the outcome of the test, the test for interfering factors should be performed again.

Procedure

(1) Gel-clot limit test

Prepare solutions A, B, C and D as shown in Table 2, and perform the test following the procedure in the *Test for confirmation of labelled TAL reagent sensitivity*.

Table 2 Preparation of solutions in gel-clot limit test

Solution	Endotoxin Concentration/Solution to which endotoxin is added	Number of replicates
A	None/Diluted test solution	2
B	2 λ /Diluted test solution	2
C	2 λ /Water for BET	2
D	None/Water for BET	2

Solution A: solution of the preparation being examined

Solution B: positive product control

Solution C: positive control

Solution D: negative control

Prepare solution A and solution B (positive product control) using a test solution at the dilution of the MVD, with which the *Test for interfering factors* was completed.

Invaluation of results The test is not valid unless both replicates of the two positive control solutions B and C are positive and those of negative control solution D are negative.

The preparation being examined complies with the test when a negative result is found for both replicates of solution A. The preparation being examined does not comply with the test when a positive result is found for both replicates of solution

A. Repeat the test by 4 replicates of solution A when a positive result is found for one replicate of solution A and a negative result is found for the other. The preparation being examined complies with the test if a negative result is found for all replicates of solution A in the repeat test.

(2) Gel-clot semiquantitative test

The test quantifies bacterial endotoxins in the test solution by titration to an endpoint. Prepare solutions A, B, C and D as shown in Table 3, and perform the test following the procedure in the *Test for confirmation of labelled TAL reagent sensitivity*.

Table 3 Preparation of solutions in the gel-clot semiquantitative test

Solution	Endotoxin Concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	Water for BET	1	—	2
			2	—	2
			4	—	2
			8	—	2
B	2 λ /Test solution	Water for BET	1	2 λ	2
C	2 λ /Water for BET		2	1 λ	2
			4	0.5 λ	2
			8	0.25 λ	2
D	None/Water for BET	—	—	—	2

Solution A: test solution at the dilution, not exceeding the MVD, with which the test for interference factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make two dilution series of 1, 1/2, 1/4, and 1/8, relative to the dilution with which the test for interfering factors was carried out.

Solution B: solution A containing standard endotoxin at a concentration of 2 λ (positive product control).

Solution C: two series of water for BET containing the standard endotoxin at concentrations of 2 λ , λ , 0.5 λ , and 0.25 λ , respectively.

Solution D: negative control (water for BET).

Invaluation of results The test is not valid unless the following 3 conditions are met: (1) both replicates of solution D (negative control) are negative; (2) both replicates of solution B (positive product control) are positive; and (3) the geometric mean endpoint concentration of solution C is in the range of 0.5 λ to 2 λ .

To determine the endotoxin concentration of solution A, calculate the endpoint concentration for each replicate series of dilutions by multiplying each endpoint dilution factor by λ . The endotoxin concentration in the test solution is the geometric mean endpoint concentration of the replicates ($E = \lg^{-1}(\sum X/2)$). If the test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution by multiplying the result by the dilution factor.

If none of the dilutions of the test solution is positive in a valid test, record the endotoxin concentration as less than λ (or, if a diluted sample was tested, as less than λ times the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is recorded as equal to or greater than the greatest dilution factor multiplied by λ .

The preparation being examined meets the requirements of the test if the concentration of endotoxin is less than that specified in the individual monograph. Otherwise, the preparation being examined does not meet the requirements of the test.

Photometric method (Method 2)

The photometric method includes a turbidimetric method and a chromogenic method.

The turbidimetric method measures the endotoxin concentrations of test solutions based on the measurement of the increase in turbidity during the gel formation of the TAL reagent. Depending on the test principle used, this method is classified as being the endpoint-turbidimetric test or the kinetic-

turbidimetric test. The endpoint-turbidimetric test is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric test is a method to measure either the onset time needed for the reaction mixture to reach a predetermined absorbance, or the rate of turbidity development.

The chromogenic method measures the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the TAL reagent. Depending on the test principle employed, this method is classified as being the endpoint-chromogenic test or the kinetic-chromogenic test. The endpoint-chromogenic test is based on the quantitative relationship between the concentration of endotoxins and the quantity of chromophore released at the end of an incubation period. The kinetic-chromogenic test is a method to measure either the onset time needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.

All photometric tests are usually carried out by special instrumentation at the incubation temperature of $37^\circ\text{C} \pm 1^\circ\text{C}$.

The quantities and volume ratios of the substance being examined and the TAL reagent, incubation time etc. employed in the test are decided according to the related instructions of instruments and the TAL reagents.

To assure the precision or validity of the turbidimetric and chromogenic tests, preparatory tests are conducted to assure that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Assurance of criteria for the standard curve The test for assurance of criteria for the standard curve must be carried out when each new batch of TAL reagent is used or any changes are made to the

experimental conditions that are likely to influence the result of the test.

Using the standard endotoxin solution, prepare at least three endotoxin concentrations (the dilution factor of adjacent concentrations is not greater than 10) to generate the standard curve within the range of endotoxin concentrations indicated by the TAL reagent manufacturer. The mixing time for every dilution is the same as that of gel-clot method. Perform the test using at least three replicates of each standard endotoxin solution, and duplicate of negative control solution at the same

time. When both reaction times of the duplicate of negative control solution are greater than that of the lowest concentration, perform statistic analysis of linear regression for all the data.

The test is not valid unless the absolute value of the correlation coefficient, $|r|$, is greater than or equal to 0.980, otherwise repeat the test.

Test for interfering factors Select an endotoxin concentration (λ_m) at or near the middle of the endotoxin standard curve. Prepare solutions A, B, C and D as shown in Table 4.

Table 4 Preparation of solutions in the test for interfering factors by using photometric method

Solution	Endotoxin Concentration	Solution to which Endotoxin is Added	Number of Replicates
A	None	Test solution	not less than 2
B	Middle concentration(λ_m) of the standard curve	Test solution	not less than 2
C	At least 3 concentrations (lowest concentration, λ , is designated)	Water for BET	each not less than 2
D	None	Water for BET	not less than 2

Solution A: test solution, that may be diluted not to exceed the MVD

Solution B: preparation to be examined at the same dilution as Solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

Solution C: standard endotoxin solution at the concentrations used in the validation of the method described in *Assurance of criteria for the standard curve*.

Solution D: water for (negative control) BET.

Calculate the content of endotoxin contained in the solution A (C_t) and solution B (C_s) respectively, and calculate the recovery of the endotoxin added to solution B as follows:

$$R = [(C_s - C_t) / \lambda_m] \times 100\%$$

If under the conditions of the test, the recovery of the endotoxin added to solution B is within 50% to 200%, the test solution is considered to be free of interfering factors.

When the endotoxin recovery is out of the specified ranges, the interfering factors must be removed as described in the *Test for interfering factors* under *Gel-Clot Techniques*. The efficiency of the treatment is verified by repeating the test for interfering factors.

When the source of the TAL reagent, or the origin, formula and the manufacture process of the substance being examined are changed, or there is any change in the experimental conditions which may affect the outcome of the test, the *Test for interfering factors* should be performed again.

Procedure

Follow the procedure described in the *Test for interfering factors* under *Photometric method*.

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the series of positive controls, solution C.

The test is not valid unless the following 3 requirements are met;

(1) the results obtained with the series of positive controls, solution C, comply with the requirements for validation defined in the *Assurance of*

criteria for the standard curve under Photometric method;

(2) the endotoxin recovery, calculated from the concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50% to 200%;

(3) both reaction times of the solution D (negative control) are greater than that of the lowest concentration of the endotoxin standard curve.

Invaluation of results The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product. Otherwise, the preparation being examined does not meet the requirements of the test.

Note In this chapter, the term "tube" includes all types of receptacles such as micro-titer plate wells.

XII F Test for Abnormal Toxicity

Abnormal toxicity test refers to the general safety test for the non-specific toxicity of the biologics. It is performed to find out any possible contamination caused by exogenous agents and those unexpected factors that may create safety problems.

Procedure

For abnormal toxicity test, both mice and guinea pigs shall be used, unless otherwise specified.

1. Test in mice

Five mice are used for the test on each batch of product, unless otherwise specified. Weigh each mouse before test. The body weight of each mouse shall be 18-22 g. Each mouse shall be injected i. p. with 0.5 ml of test sample and observed for 7 days. The product passes the test if all the mice remain healthy and survive the observation period, without any abnormal reactions, and with increase of body weight by the end of observation period. If the product fails to pass the test, the test may be repeated once by using another ten mice, and the result shall be judged as described above.

2. Test in guinea pigs

Two guinea pigs are used for the test on each batch of product, unless otherwise specified. Weigh each guinea pig before test. The body weight of each guinea pig shall be 250-350 g. Each guinea pig shall be injected i. p. with 5.0 ml of test sample and observed for 7 days. The product passes the test if all the guinea pigs remain healthy and survive the observation period, without any abnormal reactions, and with increase of body weight by the end of observation period. If the product fails to pass the test, the test may be repeated once by using another four guinea pigs, and the result shall be judged as described above.

XII G Microbial Limit Tests

Microbial limit tests provide tests for the estimation of the number of viable microorganisms present in non-sterile pharmaceutical products of all kinds, including preparations, raw materials, excipients. The bacteria, fungi or yeasts count, as well as the specified bacteria, are tested.

Microbial limit tests shall be carried out in a class 100 laminar-air-flow cabinet located within a class 10000 clean room. The whole process shall be performed under strictly aseptic conditions to avoid any microbial contamination. The cleanliness of working areas including laminar flow cabinet, working bench, background room shall be monitored regularly according to the current national standard such as the detecting method for airborne particles, airborne microbe and settling microbe in the clean room (area) of the pharmaceutical industry.

Surfactants, neutralizing agents, or inactivators do not affect the growth of microorganisms if they are used in the products to be examined.

Unless otherwise specified, incubate bacteria at 30-35°C, at 23-28°C for fungi or yeasts, and at 35-37°C for specified bacteria.

The test result is reported in the unit of 1 g, 1 ml,

10 g, 10 ml or 10 cm².

Quantity of products to be tested

It refers to the quantity of the product to be examined for one single test (in g, ml, or cm²). Unless otherwise prescribed, use samples of 10 g or 10 ml of the product to be examined for testing. Use 100 cm² for pellicles. The quantity may be appropriately decreased for expensive drugs and small-package drugs. Increase the quantity by 10 g or 10 ml when *Salmonella* is tested.

Select the samples at random from at least 2 minimum containers, and at least 4 pieces for pellicles.

The quantity of sampling (from at least 2 minimum containers) is 3 times the quantity for testing.

Preparation of the sample

Samples are prepared appropriately depending on their physico-chemical properties and biological characteristics. If necessary, samples are warmed in a water bath not more than 45°C. The samples are inoculated to the culture media within one hour of preparation.

Unless specified otherwise, follow the methods below to prepare the samples.

1. Liquids

Take 10 ml of the product, dilute to 100 ml with sterile sodium chloride-peptone buffer (pH 7.0), mix well. Use this 1 : 10 solution as the test solution. Add an appropriate amount of sterile polysorbate 80 to an oil to make it dispense evenly. For aqueous preparations, the products may be mixed as the test solution.

2. Solids, semisolids and viscous products

Take 10 ml of the product, dilute to 100 ml with sterile sodium chloride-peptone buffer (pH 7.0), mix well with a homogenizer or other devices. Use this 1 : 10 solution as the test solution. If necessary, add an appropriate amount of polysorbate 80 and warm the sample in a water bath so that it dispense evenly.

3. Sample specified preparation

(1) Non-aqueous products

Method 1 Transfer 5 g (or 5 ml) of the product into a sterile beaker containing 5 g melt (not exceeding 45°C) Span 80, 3 g glyceryl monostearate, 10 g polysorbate 80, mix, slowly add sterile sodium chloride-peptone buffer (pH 7.0, 45°C) to 100 ml with agitating to make an emulsion. Use this 1 : 20 emulsion as the test solution.

Method 2 Transfer 10 g of the product to a suitable container with 20 ml sterile isopropyl myristate (prepared as described under Sterility test, the amount may be increased if necessary) and sterile glass beads, agitate thoroughly to dissolve the product. Then add 100 ml sterile

sodium chloride-peptone buffer (pH 7.0, 45°C), agitate for 5-10 min for extraction. Allow it to stand until the two layers separate. Use the 1 : 10 aqueous layer as the test solution.

(2) Pellicles

Take 100 cm² of the pellicles, cut into pieces. Immerse into 100 ml of sterile sodium chloride-peptone buffer (pH 7.0), and agitate. Use the 1 : 10 diluent as the test solution.

(3) Enteric-coated and colonic-coated products

Transfer 10 g of the product, dilute to 100 ml with sterile phosphate buffer solution (pH 6.8 for enteric-coated and pH 7.6 for colonic-coated), warm in a 45°C water bath, and shake to dissolve. Use this 1 : 10 solution as the test solution.

(4) Aerosols and sprays

Chill a prescribed amount of the product in a refrigerator for approximately 1 hour. Take it out, dig a small hole rapidly and aseptically. Put it at room temperature and allow the propellant to escape. Transfer all the residue with a sterile syringe, add an appropriate amount of sterile sodium chloride-peptone buffer (pH 7.0, add an appropriate amount of sterile polysorbate 80 for water-insoluble ingredients), and mix well. Take a sample equivalent to 10 g or 10 ml, and dilute by 10 folds. Use this 1 : 10 solution as the test solution.

(5) Products containing antimicrobial agents

When the products to be examined possess antimicrobial activities, eliminate the activity before the test. Follow the methods described below.

① Culture media dilution Transfer the prescribed amount of the product to an adequate volume of culture media. Thus the concentration of the product decreases until it possesses no antimicrobial activity. For the limit test of bacteria, fungi, and yeasts, distribute 2 ml of the diluted solution over Petri dishes. Add agar media, mix well, and allow it to solidify. Incubate the dishes under appropriate conditions. Count the average number of colonies obtained with 1 ml of the solution. Report the counts by the plate-count method. The amount of diluting culture media may increase when specified bacteria are tested.

② Enrichment of microorganisms by centrifugation

Centrifuge a specified quantity of the product at 3000 r/min for 20 minutes. If the product contains precipitates, centrifuge at 500 r/min for 5 minutes at first, and then collect the supernatant and centrifuge again as described above. Discard the supernatant and dilute the residue at the bottom (about 2 ml) to original specified quantity with diluting solution.

③ Membrane filtration See the membrane filtration method under Bacteria, fungi, and yeasts count.

④ Neutralization If the product to be examined

contains antimicrobial agents like mercury, arsenic, or antiseptics, use suitable neutralizing agents or inactivators to eliminate its antimicrobial activity. The neutralizing agents or inactivators may be added in the diluting solutions or culture media.

Bacteria, fungi or yeasts count

Method validation

The bacteria, fungi or yeasts count method for microbial limit tests is validated before it is used for drugs. The count method is revalidated if changes in composition of the product or testing conditions may affect the result.

Validation test is to be conducted as described under Bacteria, fungi or yeasts count using exactly the same methods and the following instructions. The tests should be performed separately for each of the microorganism tested.

Test strains

The viable microorganisms used in the test must be not more than five passages removed from the original master seed lot. The suitable seed-stock technique should be used so that the microorganism characters can be maintained.

<i>Escherichia coli</i>	[CMCC (B) 44102]
<i>Staphylococcus aureus</i>	[CMCC (B) 26003]
<i>Bacillus subtilis</i>	[CMCC (B) 63501]
<i>Candida albicans</i>	[CMCC (F) 98001]
<i>Aspergillus niger</i>	[CMCC (F) 98003]

Preparation of Inoculum Inoculate freshly cultured of *Escherichia coli*, *Staphylococcus aureus*, or *Bacillus subtilis* to nutrient broth medium or nutrient agar medium, incubate for 18-24 hours; Inoculate freshly cultured of *Candida albicans* to modified Martin medium or modified Martin agar medium, and incubate for 24-48 hours; The above cultures are diluted to 50-100 colony-forming units (CFU) per ml with sterile solution of 0.9% sodium chloride. Inoculate freshly cultured of *Aspergillus niger* to modified Martin agar medium, and incubate for 5-7 days. Wash out the spores with 3-5 ml sterile solution of 0.9% sodium chloride. Transfer the spore suspension to a sterile test tube. Dilute the suspension to 50-100 CFU per ml with sterile solution of 0.9% sodium chloride.

Validation test Carry out at least 3 parallel tests, and calculate the recovery of microorganisms for each test.

(1) Testing group When the plate count method is used, transfer 1 ml of the lowest possible serial dilution of the sample and 50-100 CFU testing microorganisms to a Petri dish, and pour the agar medium immediately. Use 2 Petri dishes for each microbial strain, and record the colony number by the plate count method. When the membrane filtration method is used, transfer specified quantity of the sample solution through a membrane, rinse appropriately, add 50-100 CFU

testing microorganisms in the last portion of rinsing solution. Record the colony number by membrane filtration method.

(2) Microbial group Determine the number of microorganisms added in the test.

(3) Product control Transfer a prescribed quantity of the product, determine the number of bacteria, fungi or yeasts in sample.

(4) Diluting solution control Diluting solution control should be carry out when the sample of preparation used dispersant, emulsification, neutralization, centrifugation and filtration etc. Use diluting solution instead of sample and add the testing microorganisms to 50-100 CFU per ml, preparation test solution and determine the number of microorganisms as described above for testing group.

Evaluation of results

In each of the 3 parallel tests, the microbial recovery is not less than 70% (average colony number of the diluting solution control/average colony number of the testing group $\times 100\%$). If the microbial recovery of the testing group is not less than 70% [(average colony number of the testing group-average colony number of the product control)/average colony number of the microbial group $\times 100\%$], proceed the bacteria, fungi or yeasts count of the product to be examined as described above. If the microbial recovery in any of the tests is below 70%, eliminate the antimicrobial activities of the product by appropriate methods like culture media dilution, centrifugation, membrane filtration, or neutralization. The method needs re-validation.

The validation test is carried out in parallel with the bacteria, fungi, or yeasts count of the product to be examined.

Examination method

Examination method include plate count and membrane filtration method. Use validated plate count method or membrane filtration method to carry out the bacteria, fungi, or yeasts count of the product to be examined.

Dilute the homogenized solution to be test with pH 7.0 sterile solution of sodium chloride-peptone buffer solution to make serial dilutions of 1 : 10, 1 : 100, 1 : 1000, etc.

1. Plate count method

Use two to three successive serial dilutions as the testing solution. Transfer 1 ml of the sample to a sterile Petri dish (90 mm in diameter), add 15-20 ml Nutrient agar medium, or Sodium rose bengal agar medium, or Yeast extracts peptone glucose agar medium (melted at not exceeding 45°C), mix well, and allow the contents to solidify at room temperature. Invert the Petri dish and incubate under appropriate conditions. For each dilution, use at least 2 Petri dishes for each culture medium.

Negative control Transfer 1 ml of the dilution to a sterile Petri dish, promptly add the culture medium, and allow the contents to solidify at room temperature. Invert the Petri dish and incubate under appropriate conditions. Use at least 2 Petri dishes for each culture medium. No evident growth of microorganisms occurs in either of the Petri dish.

Incubation and counting Unless otherwise prescribed, incubate the bacteria for 48 hours, count the number of colonies every day, report the number on the 48 hour; incubate the fungi and yeasts for 72 hours, count the number of colonies every day, report the number on the 72 hour; extend the incubation time to 5-7 days if necessary. Do not count the number if colonies assemble. Following counting, calculate the number of colonies of each dilution of the product, report the result following the Microbial number report rule described below. For liquid preparations containing honey and royal jelly, use Sodium rose bengal agar medium for fungi count and Yeast extracts peptone glucose agar medium for yeasts count. Sum the counts as the final result.

Microbial number report rule Select the dilution in which the average number of colonies of bacteria and yeasts is 30-300 CFU and of the fungi is 30-100 CFU.

If average microbial number of any dilution is less than 30, select the number of the lowest dilution. Calculate the number of CFU per g or per ml product to be tested.

When no microbial growth occurs in any of the dilutions, or it only occurs for the lowest dilution where the average microbial number of the CFU is less than 1, report the result with 1 multiplying by the lowest dilution folds.

2. Membrane filtration

Use membranes having a nominal pore size not greater than 0.45 μm , and a diameter of appropriately 50 mm. The type of filter material is chosen in such a way that the bacteria retaining efficiency is not affected by the component or solvent of the sample to be investigated. The filter unit and membrane are sterilized prior to use by appropriate means. Maintain the performance characteristic of the filter during the testing process. Moist the membrane with a minimum amount of rinsing solution before the test of aqueous products. Where the product to be examined is an oil, the membrane and filter unit are thoroughly dried before use. Let the product solution and rinsing solution cover the whole membrane to obtain its maximal performance. Rinse the membrane with 100 ml of rinsing solution each time if necessary. Do not use a large amount of rinsing solutions to avoid disturbance of the microorganisms on the membrane.

Take representing 1 g or 1 ml of the product, or 1 ml suitable dilution solution of the sample if

product include large numbers of microorganism), and dilute to 100 ml with diluting solution, mix well and filter. Rinse the membrane with pH 7.0 sterile sodium chloride-peptone buffer solution or other suitable rinsing solution, as described above for validation test. Transfer the membrane onto a Nutrient agar medium or Sodium rose bengal agar medium, or Yeast extracts peptone glucose agar medium plate, and incubate. Use at least one membrane for each medium.

Negative control Use 1 ml of the dilution, and carry out the test as described above. No evident growth of microorganisms occurs in the negative control.

Incubation and counting Carry out the test as described above for plate count method. The number of microorganisms on each membrane is not more than 100.

Microbial number report rule Multiply the average microbial number by dilution folds as the number of CFU per g or per ml product to be test. If no microbial growth occurs, report the result with less than 1 or 1 multiplying by the lowest dilution folds.

Test for specified microorganisms

Method validation

The method for specified microorganisms test is validated before it is used for microbial limit tests for drugs. The method is revalidated if changes in composition of the product or testing conditions may affect the result.

Validation microorganisms used shall be complied with requirements of the microbial limits of the product. Validation the method of coliform should be used *Escherichia coli*. Carry out the test as described under the Test for specified microorganisms exactly the same methods and the following instructions.

Test strains he strains comply with the requirements as described above for bacteria, fungi, or yeasts count.

<i>Escherichia coli</i>	[CMCC (B) 44102]
<i>Staphylococcus aureus</i>	[CMCC (B) 26003]
<i>Salmonella paratyphi B</i>	[CMCC (B) 50094]
<i>Pseudomonas aeruginosa</i>	[CMCC (B) 10104]
<i>Clostridium sporogenes</i>	[CMCC (B) 64941]

Preparation of Inoculum Inoculate freshly cultured of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella paratyphi B*, or *Pseudomonas aeruginosa* to nutrient broth medium or nutrient agar medium; inoculate freshly cultured of *Clostridium sporogenes* to fluid thioglycollate medium; incubate for 18-24 hours. Dilute the suspension to 10-100 CFU per ml with sterile solution of 0.9% sodium chloride.

Validation test

(1) Testing group Add a specified quantity of the product to be examined and 10-100 CFU testing

microorganisms to the culture medium, carry out the test by prescribed methods described below. When the membrane filtration method is used, filter an appropriate quantity of the product through a membrane, rinse appropriately, add the testing microorganisms in the last portion of rinsing solution. Transfer the membrane to the culture medium for incubation.

(2) Negative control Negative control is set to verify the specificity of the testing method. The procedure is the same as described above for testing group. Use *Staphylococcus aureus* as the negative control microorganism for the test of *Escherichia coli*, coliform, or *Salmonella* species. Use *Escherichia coli* as the negative control microorganism for the test of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Clostridium* species. No control microorganism is detected.

Evaluation of results

No control microorganism is detected in the negative control. If the tested microorganism is detected in the testing group, proceed the test with the product to be examined. If the tested microorganism is not detected in the testing group, eliminate the antimicrobial activities of the product by appropriate methods like culture media dilution, centrifugation, membrane filtration, or neutralization. The method needs revalidation. The validation test is carried out in parallel with the test for specified microorganisms of the product to be examined.

Examination method

Carry out the test of the product to be examined as described above for validation test.

Positive control Add 10-100 CFU of the tested microorganism to the positive control, carry out the test as the Test for specified microorganisms. The tested microorganism can be detected in the positive control.

Negative control Transfer 10 ml of diluting solution to a prescribed amount of culture media, and incubate as the Test for specified microorganisms. No microorganism growth occurs in the negative control.

(1) *Escherichia coli* Inoculate 10 ml of the test solution (equivalent to 1 g, 1 ml or 10 cm²) to be examined to an appropriate amount of Bile salt lactose culture medium (not less than 100 ml) directly or after appropriate treatment, incubate for 18-24 hours (48 hours if necessary). Inoculate 0.2 ml of the above culture to a tube containing 5 ml MUG medium, and incubate. Observe under 366 nm UV light at the time of 5 hours and 24 hours, respectively. Use blank MUG medium as the negative control. The result is MUG-positive if the cultures give fluorescent light, or MUG negative if no fluorescent light is observed. Following observation, add several

drops of indole test solution. The result is indole-positive if the liquid surface presents a rosy colour, or indole-negative if no colour change takes place. The negative control is MUG-negative and indole-negative.

A MUG-positive and indole-positive result indicates the presence of *E. coli* in the product. A MUG-negative and indole-negative result indicates the absence of *E. coli* in the product. If the product is MUG-positive and indole-negative, or MUG-negative and indole-positive, inoculate the cultures on eosin methylene blue agar medium plate or MacConkey agar medium plate, and incubate for 18-24 hours.

If no growth of microorganism occurs on the plate, or the appearance of the microbial colonies does not match the descriptions in Table 1, the product passes the test. Otherwise, confirm the result by suitable biochemical tests.

Table 1 Morphologic characteristics of *Escherichia coli* colonies

Culture medium	Characteristic colonial morphology
Eosin methylene blue agar medium	Purple black, light purple, bluish purple or pink, deep purple at the center of colony or no obvious dark center, circular, slight convex, regular margin, smooth surface, moist, metal gloss often appeared.
MacConkey agar medium	Brilliant pink or pale red, deep pink at center of the colony, circular, flat, regular margin, smooth surface, moist.

(2) Coliform Use 3 tubes each containing an appropriate amount of Bile salt lactose fermentation culture medium (not less than 10 ml), respectively add 1 ml of 1 : 10 (containing 0.1 g or 0.1 ml of the product), 1 : 100 (containing 0.01 g or 0.01 ml of the product), 1 : 1000 (containing 0.001 g or 0.001 ml of the product) dilutions. Add 1 ml of diluting solution to another tube as the negative control. Incubate for 18-24 hours.

The product passes the test if no microbial growth occurs, or no gas bubbles or acid forms. If the formation of acid and gas bubbles is observed, inoculate the cultures on Eosin methylene blue agar medium plate or MacConkey agar medium plate, and incubate for 18-24 hours.

If no growth of microorganisms occurs on the plate, or the appearance of the microbial colonies does not match the descriptions in Table 2, or the colonies are not Gram-negative bacilli, the product passes the test. If the morphology of the colonies matches the descriptions in Table 2, and they are Gram-negative bacilli without spores, confirm the result by suitable biochemical tests.

Validation test Choose 4-5 suspect colonies from the plate, individually inoculate in tubes containing bile salt lactose culture medium, and incubate for 24-48 hours. The formation of acid and gas bubbles indicates the presence of coliform.

Otherwise, absence coliform in the product.

Table 2 Morphologic characteristics of coliform colonies

Culture medium	Characteristic colonial morphology
Eosin methylene blue agar medium	Purple black, or purple red, circular, slight convex, regular margin, smooth surface, moist.
MacConkey agar medium	Brilliant pink or pale red, circular, flat, regular margin, smooth surface, moist.

According to the number of coliform-positive tubes, and Table 3, record the probable number of coliform 1 g or 1 ml of the product.

Table 3 Probable number of coliform

Result of each quantity of product			Probable number of coliform N (per g or ml)
0.1 g or 0.1 ml	0.01 g or 0.01 ml	0.001 g or 0.001 ml	
+	+	+	$N > 10^3$
+	+	-	$10^2 < N < 10^3$
+	-	-	$10 < N < 10^2$
-	-	-	$N < 10$

Note: + represents coliform is detected, and - not detected.

(3) *Salmonella* species Inoculate 10 g or 10 ml of the product to be examined to an appropriate amount (not less than 200 ml) of Nutrient broth culture medium directly or after appropriate treatment, mix well with a homogenizer or other devices, and incubate for 18-24 hours.

Inoculate 1 ml of the culture above to 10 ml Sodium tetrathionate brilliant green culture medium, incubate for 18-24 hours. Then streak the cultures on the surface of Bile salt sulfur milk agar culture medium (or *Salmonella* and *Shigella* agar medium) and MacConkey agar medium (or Eosin methylene blue agar) plates separately, incubate for 18-24 hours (or 40-48 hours if necessary). If no bacterial growth occurs on the plate, or the morphology of the colonies does not conform to the descriptions in Table 4, *Salmonella* is absence in the product to be tested.

If the morphology of the colonies conforms to or is similar to the description in Table 4, choose 2-3 colonies and transfer to Triple sugar iron agar culture medium slant with a inoculating needle, using surface and deep inoculation. Incubate for 18-24 hours. If red colour on the surface and yellow colour at the bottom, or yellow on the surface and black at the bottom is not observed, *Salmonella* is absence in the product to be tested. Otherwise, confirm the presence of *Salmonella* by carrying out suitable biochemical and Serum agglutination tests with the cultures on triple sugar iron agar culture medium slant.

Table 4 Morphologic characteristics of *Salmonella* colonies

Culture medium	Characteristic colonial morphology
Bile salt sulfur lactose agar	Colourless to pale orange, semitransparent, black at the center or whole black or no black
Salmonella and Shigella agar medium	Colourless to pale red, semitransparent or opaque, black brown in the center of the colonies occasionally
Eosin methylene blue agar medium	Colourless to pale orange colour, transparent or semitransparent, smooth moist circular colonies
MacConkey agar medium	Colourless to pale orange colour, transparent or semitransparent, dark in the center occasionally

(4) *Pseudomonas aeruginosa* Inoculate 10 ml of the test solution (equivalent to 1 g, 1 ml or 10 cm² of the product) to an appropriate amount (not less than 100 ml) of Bile salt lactose culture medium, incubate for 18-24 hours. Then streak the cultures on the surface of Cetyl trimethylammonium bromide agar culture plate, and incubate for 18-24 hours.

Pseudomonas aeruginosa colonies are typically flat, irregular, diffusing margin, moist surface, greyish white, occasionally surrounded by bluish green zone. If no bacterial growth occurs on the plate, or the morphology of the colonies does not conform to the above descriptions, *Pseudomonas aeruginosa* is absence in the product to be tested. If the growth colonies reveal the above characters, choose 2-3 colonies, inoculate separately on Nutrient agar culture slant, incubate for 18-24 hours. Use the slant cultures for Gram's staining, microscopical examination, and oxidase test.

Oxidase test Place a piece of clean filter paper in a Petri dish, spread the slant cultures on the filter paper with a sterile glass bar, add dropwise freshly prepared 1% *N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride test solution. The oxidase test is positive if the cultures appear pink and then turn into purplish red within 30 seconds. Otherwise, the test is negative.

If the slant cultures are not Gram-negative bacilli, or the oxidase test is negative, *Pseudomonas aeruginosa* is absence in the product to be tested. Otherwise, carry out the pyocyanin test.

Pyocyanin test Inoculate the slant cultures onto PDP agar culture slant, and incubate for 24 hours. Then add 3-5 ml of chloroform to the test tube, agitate the medium and shake thoroughly. Allow to stand for a while, then transfer the chloroform layer to a new tube, add about 1 ml of 1 mol/L hydrochloric acid solution, allow to stand for a moment following shaking, observe the phenomenon. If the solution appears pink, the pyocyanin test is positive; or otherwise, negative. Use uninoculated PDP agar culture slant as the negative control. The negative control is pyocyanin-negative.

If the bacteria in question are Gram-negative bacilli, and are oxidase-test positive and pyocyanin-test positive, *Pseudomonas aeruginosa* is presence in the product to be examined. If the bacteria in question are Gram-negative bacilli, and are oxidase-test positive and pyocyanin-test negative, proceed the test by carrying out suitable biochemical tests to confirm whether they are *Pseudomonas aeruginosa*.

(5) *Staphylococcus aureus* Inoculate 10 ml of the test solution (equivalent to 1 g, 1 ml or 10 cm² of the product) to an appropriate amount (not less than 100 ml) of Sodium tellurite broth culture medium (or Nutrient broth culture medium) directly or after appropriate treatment, incubate for 18-24 hours (48 hours if necessary). Then streak the cultures on the surface of Egg yolk high salt agar culture medium plate or Mannitol high salt agar culture medium plate, incubate for 24-72 hours. If no bacterial growth occurs on the plate, or the morphology of the colonies does not conform to the descriptions in Table 5, *Staphylococcus aureus* is absence in the product in question.

Table 5 Morphologic characteristics of *Staphylococcus aureus* colonies

Culture medium	Characteristic colonial morphology
Mannitol high salt agar culture medium	Golden yellow, circular convex, regular margin, yellow outer circle, colony diameter 0.7-1 mm.
Egg yolk high salt agar culture medium	Golden yellow, circular convex, regular margin, opaque outer circle due to lecithin degradation, colony diameter 1-2 mm.

If the morphology of the colonies conforms to or is similar to the description in Table 5, choose 2-3 colonies and inoculate onto Nutrient agar culture medium slant, and incubate for 18-24 hours. Take the obtained cultures for Gram's staining. And inoculate the cultures in Nutrient broth culture medium, and incubate for 18-24 hours, then carry out the coagulase test.

Coagulase test Use 3 sterile tubes, add 0.5 ml of (1:1) mixture of plasma and sterile water in each tube, then respectively add 0.5 ml of nutrient broth cultures of the suspect bacteria (or concentrated bacterial suspension prepared from the nutrient agar slant cultures), 0.5 ml of nutrient broth cultures of *Staphylococcus aureus* (or concentrated bacterial suspension prepared from the nutrient agar slant cultures), and 0.5 ml of Nutrient broth culture medium or 0.9% sterile sodium chloride solution, which are testing tube, positive tube, and negative tube, respectively. Incubate the 3 tubes together. Examine the tubes at 3 hours and subsequently at suitable intervals up to 24 hours. The plasma in the negative tube maintains fluidity, and the plasma coagulates in the positive tube. If the plasma in the testing tube coagulates, the coagulase test is positive, or otherwise, negative. If the result of positive or

negative tube does not conform to the above description, repeat the test.

If the suspect bacteria are not Gram-positive cocci, and the coagulase test is negative, *Staphylococcus aureus* is absence in the product to be tested.

(6) Clostridium Take two equal portions (10 ml, equivalent to 1 g or 1 ml of the product) of the sample solution. Heat one portion at 80°C for 10 minutes and cool rapidly. Do not heat the other portion. Inoculate the test solutions above to 100 ml of Chopped meat culture medium directly or after appropriate treatment separately. Incubate under anaerobic conditions for 72-96 hours. If no turbidity, gas bubbles, beef digestion, or bad smells form in the testing tube, Clostridium is absent in the product being examined. Otherwise, spread 0.2 ml of the cultures onto the surface of columbia agar culture medium plate to which gentamicin has been added, and incubate under anaerobic conditions for 48-72 hours. If no microbial growth occurs on the plate, Clostridium is absent in the product to be tested. If colonies are observed on the plate, select 2-3 colonies and carry out Gram's staining test and catalase test.

Catalase test Put the colonies from the plate above onto clean glass slices, add dropwise 3% hydrogen peroxide solution. If gas bubbles appear on the colony surface, the catalase test is positive, or otherwise, negative.

If the suspect colonies are Gram-positive Clostridium, with or without oval or round spores, and catalase test is negative, Clostridium is presence in the product being examined. Or otherwise, Clostridium is absence.

Evaluation of results.

If specified microorganisms or other pathogenic microorganisms are detected in one single test, the product does not pass the test. The test needs not repetition.

If any count of bacteria, of fungi, or of yeasts does not comply with the requirements, take samples at random from the same batch, and carry out two more independent repetitive tests. Report the average value from three determinations as the final result.

If fungi and yeasts are detected in eye preparations, the product being examined passes the test for fungi and yeasts count only if microorganisms are not detected in two more repetitive tests.

If results of bacteria, fungi and yeasts count, as well as the test for specified microorganisms meet the specified requirements for the product being examined, the product passes the test. If the result of bacteria, fungi and yeasts count, or the result for the test for specified microorganisms does not comply with the specified requirements for the product being examined, the product does not pass the test.

Diluting solutions

The diluting solutions are sterilized by validated sterilization methods after preparation.

1. pH 7.0 sterile sodium chloride-peptone buffer solution

Prepare the solution as described under the Test for Sterility (Append XII H, Volume II)

2. pH 6.8 sterile phosphate buffer solution, pH 7.6 sterile phosphate buffer solution

Prepare the solution as described under Appendix XV D of Volume II, and then filter, dispense in containers, and sterilize.

If necessary, add surfactants or neutralizing solutions before or after the sterilization.

3. 0.9% sterile sodium chloride solution

Transfer 9.0 g sodium chloride, dissolve in 1000 ml water, filter, dispense in containers, and sterilize.

Culture media

The media may be prepared as described below, or their dehydrated formulations may be used. The media should be sterilized using a validated process.

1. Nutrient agar culture medium, nutrient broth culture medium, fluid thioglycollate medium, modified Martin medium, and modified Martin agar medium

Prepare the media as described under the Test for Sterility (Appendix XII H, Volume II).

2. Sodium rose bengal agar medium

Peptone	5.0 g
Glucose	10.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.5 g
Sodium tetrachloro-tetraiodo-fluorescein	0.0133 g
Agar	14.0 g
water	1000 ml

Mix the above ingredients in water except glucose and sodium tetrachloro-tetraiodo-fluorescein, heat until dissolved, filter, and then add glucose and sodium tetrachloro-tetraiodo-fluorescein, Dispense in containers and sterilize.

3. Yeast extracts peptone glucose agar medium (YPD)

peptone	10.0 g
Yeast extracts	5.0 g
Glucose	20.0 g
agar	14.0 g
water	1000 ml

Mix the above ingredients in water except glucose, heat until dissolved, filter, and then add glucose, Dispense in containers and sterilize.

4. Bile salts lactose medium (BL)

peptone	20.0 g
lactose	5.0 g
Sodium chloride	5.0 g

Dipotassium hydrogen phosphate (K_2HPO_4)

4.0 g

Potassium dihydrogen phosphate (KH_2PO_4)

1.3 g

Ox bile salts (or sodium desoxycholate 0.5 g)

2.0 g

water

1000 ml

Mix the above ingredients in water except lactose and ox bile salts (or sodium desoxycholate), heat until dissolved, adjust the pH so that after sterilization it is 7.4 ± 0.2 , boiling. Filter, add lactose and ox bile salts (or sodium desoxycholate), dispense in containers and sterilize.

5. Bile salts lactose fermentation medium

Add 0.04% bromocresol purple indicator to the unsterilized bile salts lactose medium, dispense in suitable tubes containing inverted tubule, and sterilize.

6. Eosin methylene blue agar medium (EMB)

Nutrient agar medium

100 ml

2% eosin solution

2 ml

20% lactose solution

5 ml

0.5% methylene blue solution

1.3-1.6 ml

Melt the nutrient agar medium by heating, cool to $60^\circ C$, add aseptically the other three solutions, shake thoroughly, and pour into Petri dishes.

7. MacConkey agar medium (MacC)

Peptone

20.0 g

Sodium chloride

5.0 g

Lactose

10.0 g

1% neutral red solution

3.0 ml

Ox bile salts

5.0 g

Agar

14.0 g

Water

1000 ml

Mix peptone and sodium chloride in water, heat until dissolved, adjust the pH so that after sterilization it is 7.2 ± 0.2 . Add agar, heat to melt, then add the other ingredients. Shake thoroughly, dispense in containers, and sterilize. Cool to $60^\circ C$, and pour into the Petri dishes.

8. 4-methylumbelliferyl- β -D-glucuronide (MUG) culture medium

Peptone

10.0 g

Manganese sulfate

0.5 mg

Zinc sulfate

0.5 mg

Magnesium sulfate

0.1 g

Sodium chloride

5.0 g

Calcium chloride

50 mg

Potassium dihydrogen phosphate (anhydrous)

0.9 g

Disodium hydrogen phosphate (anhydrous)

6.2 g

Sodium sulfite

40 mg

Sodium desoxycholate

1.0 g

MUG

75 mg

Water

1000 ml

Mix the above ingredients in water except MUG, heat until dissolved, adjust the pH so that after sterilization it is 7.3 ± 0.1 , add MUG, dissolve, dispense 5 ml of the medium to each tube and

sterilize.

9. Triple sugar iron agar medium (TSI)

Peptone

20.0 g

Glucose

1.0 g

Beef extract powder

5.0 g

Sodium chloride

5.0 g

Lactose

10.0 g

Sucrose

10.0 g

Ferrous sulfate

0.2 g

Sodium thiosulfate

0.2 g

0.2% phenosulfonphthalein solution

12.5 ml

Agar

12.0 g

Water

1000 ml

Mix peptone, beef extracts powder, sodium chloride, ferrous sulfate and sodium thiosulfate, dissolve in water heat until dissolved, adjust the pH so that after sterilization it is 7.3 ± 0.1 . Add agar, heat to melt, then add the other ingredients. Shake thoroughly, dispense in containers, and sterilize. Make short slants (2-3 cm) after the media cool.

10. Sodium tetrathionate brilliant green medium (TTB)

Peptone

5.0 g

Calcium carbonate

10.0 g

Ox bile salts

1.0 g

Sodium thiosulfate

30.0 g

Water

1000 ml

Dissolve the above ingredients in water heat until dissolved, and sterilize.

Just prior to use, add 0.2 ml iodine test solution and 0.1 ml brilliant green test solution to 10 ml of the medium, and shake thoroughly.

11. Salmonella and Shigella agar culture medium (SS)

Peptone

5.0 g

Sodium citrate

8.5 g

0.1% Brilliant green solution

0.33 ml

Beef extract powder

5.0 g

Ammonium ferrous citrate

1.0 g

Lactose

10.0 g

Sodium thiosulfate

8.5 g

Ox bile salts

8.5 g

1% neutral red solution

2.5 ml

Agar

16.0 g

Water

1000 ml

Mix the above ingredients in water except lactose, neutral red solution and agar, heat until dissolved, adjust the pH so that after sterilization it is 7.2 ± 0.1 . Filter, add agar and heat to melt. Then add the other ingredients, shake thoroughly, sterilize, cool to $60^\circ C$, and pour into Petri dishes.

12. Bile salts sulfur lactose agar culture medium (DHL)

Peptone

20.0 g

Beef extracts

3.0 g

Lactose

10.0 g

Sucrose

10.0 g

Sodium desoxycholate

1.0 g

Sodium thiosulfate

2.3 g

Sodium citrate	1.0 g
Ammonium ferrous citrate	1.0 g
1% neutral red solution	3 ml
Agar	16.0 g
Water	1000 ml

Mix the above ingredients in water except sugars, indicators and agar, heat until dissolved, adjust the pH so that after sterilization it is 7.2 ± 0.1 . Add agar and heat to melt. Then add the other ingredients, shake thoroughly, sterilize, cool to 60°C , and pour into Petri dishes.

13. Cetrimide agar medium

Peptone	10.0 g
Beef extracts powder	3.0 g
Sodium chloride	5.0 g
Cetyl trimethyl ammonium bromide	0.3 g
Agar	14.0 g
Water	1000 ml

Mix the above ingredients in water except agar, heat until dissolved, adjust the pH so that after sterilization it is 7.2 ± 0.1 . Add agar and heat it to melt, shake thoroughly. Dispense in containers, sterilize, cool to 60°C , and pour into Petri dishes.

14. Tellurite broth medium

Just prior to use, add 0.2 ml of freshly prepared 1% sodium (or potassium) tellurite solution, mix well.

15. Egg yolk high salts agar medium

Peptone	6.0 g
Beef extracts	1.8 g
Sodium chloride	30.0 g
10% sodium chloride egg yolk solution	100 ml
Agar	16.0-18.0 g
Water	650 ml

Mix the above ingredients in water except 10% sodium chloride egg yolk solution, heat until dissolved, adjust the pH so that after sterilization it is 7.6 ± 0.1 . Sterilize and cool to 60°C . Add aseptically 10% sodium chloride egg yolk solution, shake thoroughly, and pour into Petri dishes.

16. Mannitol high salts agar medium

Peptone	10.0 g
Beef extracts	1.0 g
Mannitol	10.0 g
Sodium chloride	75.0 g
1% phenolsulfonphthalein solution	2.5 ml
Agar	14.0 g
Water	1000 ml

Mix the above ingredients in water except mannitol, phenolsulfonphthalein solution and agar, heat until dissolved, adjust the pH so that after sterilization it is 7.6 ± 0.1 , add agar and heat to melt. Filter and dispense in containers, sterilize and cool to 60°C , then pour into Petri dishes.

17. Lactose fermentation culture medium

Peptone	20.0 g
Lactose	10.0 g
0.04% bromocresol purple indicator	25 ml

water	1000 ml
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Mix the above ingredients in water except 0.04% bromocresol purple indicator, heat until dissolved, adjust the pH so that after sterilization it is 7.2 ± 0.2 , add the indicator, dispense in suitable tubes (3 ml per tube). Sterilize.

18. Pyocyanin culture medium

Peptone	20.0 g
Magnesium chloride (anhydrous)	1.4 g
Potassium sulfate (anhydrous)	10.0 g
Glycerol	10 ml
Agar	14.0 g
Water	1000 ml

Mix peptone, magnesium chloride and potassium sulfate, heat until dissolved, adjust the pH so that after sterilization it is 7.3 ± 0.1 , add glycerol and agar, heat to melt, shake thoroughly, dispense in suitable tubes and sterilize. Cool at room temperature to make slants.

19. Chopped meat culture medium

Preparation of the Beef bits. Use fresh beef, remove the fats and muscles, and boil for 10 minutes. Cut into pieces of 5 mm^3 , add three folds (w/w) of distilled water, immerse in $4-10^{\circ}\text{C}$ water for 18-20 hours, then boil for 1 hour, filter through gauze. The residue is washed twice with water. Then put the beef bits into an appropriate amount of sodium hydroxide solution so that the pH is around 8.4, agitate thoroughly. Immerse the beef overnight, and discard the upper water the next day, and wash 2-3 times with water. Spread the beef on a tray, sterilize, and dry at $80-100^{\circ}\text{C}$, remove the powders, put into bottles, and keep away from moisture.

Preparation of chopped meat culture medium. Put the beef bits in suitable containers, add an appropriate amount of Nutrient broth medium so that the beef bits constitute 1.5% of the volume, adjust the pH so that after sterilization it is 7.3 ± 0.1 , sterilize.

20. Columbia agar culture medium

Pancreatic digest of casein	10.0 g
Pepsin digest of beef	5.0 g
Pancreatic digest of heart	3.0 g
Yeast extracts powder	5.0 g
Corn flour	1.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 ml

Mix the above ingredients in water except agar, heat until dissolved, adjust the pH so that after sterilization it is 7.3 ± 0.2 , add agar and heat to melt. Filter, dispense in containers and sterilize. Cool to $45-50^{\circ}\text{C}$, add sterile gentamicin sulfate (equivalent to 20 mg gentamicin), mix well, and pour into Petri dishes.

Microbial contamination limits of Pharmaceutical Preparations

The microbial contamination limits of non-sterile

preparations is drawn by fully considering the drug administration routes and potential harm to the patients. Unless otherwise prescribed, Microbial limit test follows the criterion below in the manufacturing, storage and distribution of preparations, as well as in the establishment of new preparations standards, evaluation of imported preparations, and the quality control of preparations, raw materials, and excipients.

1. Sterile preparations specified in General Notice or monographs, and other preparations labelled sterile
Comply with the test for Sterility.

2. Preparations for oral administration

Bacteria count not more than 1000 per g, or 100 per ml.

Fungi and yeasts count not more than 100 per g or per ml.

E. coli. Absence in 1 g or 1 ml.

3. Preparations for local administration

(1) Local administration preparations for surgery, burn or serious injury

Comply with the test for sterility.

(2) Preparations for eye administration

Bacteria count Not more than 10 per g or per ml.

Fungi and yeasts count Absence in 1 g or 1 ml.

Staphylococcus aureus, *Pseudomonas aeruginosa* and *Escherichia coli* Absence in 1 g or 1 ml.

(3) Preparations administered via ear, nose or respiratory tract

Bacteria count Not more than 100 per g, per ml or per 10 cm².

Fungi and yeasts count Not more than 10 per g, per ml or per 10 cm².

Staphylococcus aureus and *Pseudomonas aeruginosa*
Absence in 1 g, 1 ml or 10 cm².

Escherichia coli. Absence in 1 g, 1 ml or 10 cm² for preparations for nose and respiratory tract administration.

(4) Preparations for vagina and urethra administration

Bacteria count Not more than 100 per g or per ml.

Fungi and yeasts count less than 10 per g or per ml.

Staphylococcus aureus and *Pseudomonas aeruginosa*
Absence in 1 g or 1 ml.

(5) Preparations for rectal administration

Bacteria count Not more than 1000 per g, and not more than 100 per ml.

Fungi and yeasts count Not more than 100 per g or per ml.

Staphylococcus aureus, *Pseudomonas aeruginosa* and *Escherichia coli* Absence in 1 g or 1 ml.

(6) Other preparations for local administration

Bacteria count Not more than 100 per g, per ml or per 10 cm².

Fungi and yeasts count Not more than 100 per g,

per ml or per 10 cm².

Staphylococcus aureus and *Pseudomonas aeruginosa*
Absence in 1 g, 1 ml or 10 cm².

4. Oral preparations containing animal tissues (including extracts)

Salmonella species are absence per 10 g or per 10 ml.

5. Preparations for more than one administration routes

Comply with the requirements for each administration route.

6. Raw materials and excipients

Refer to the limit for corresponding preparation.

XII H Test for Murine Virus

Murine monoclonal antibodies may be potentially contaminated with viruses such as epidemic hemorrhagic fever virus, lymphocytic choriomeningitis virus, respiratory enteric orphan virus type 3, Sendai virus, mouse pox virus, mouse adenovirus, mouse pneumonia virus and retrovirus, etc. The viruses are classified as groups I and II. The viruses in group I, including epidemic hemorrhagic fever virus, lymphocytic choriomeningitis virus, respiratory enteric orphan virus type 3 and Sendai virus, can infect humans and primates. The viruses in group II, including mouse pox virus, mouse adenovirus, mouse pneumonia virus and retrovirus, so far there is no evidence that they can infect humans, but can replicate in vitro in the cell cultures of human, simian and monkey origins and may be potentially dangerous to humans, thus shall be monitored emphatically.

The method is applied to the test for the presence of murine viruses in hybridoma cell lines and murine monoclonal antibodies. The live virus antigen and antibody are tested by in vitro infectivity assay, animal antibody production test, infectivity assay in embryonated eggs, and so on.

Reagent

(1) 0.01 mol/L pH 7.4 PBS; Dissolve 2.9 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 0.2 g of sodium dihydrogen phosphate, 8.0 g of sodium chloride, 0.2 g of potassium chloride in water and dilute to 1000 ml.

(2) pH 9.6 coating buffer solution; Dissolve 1.59 g of sodium carbonate, 2.93 g of sodium bicarbonate and 0.20 g of sodium azide in water and dilute to 1000 ml.

(3) 0.01 mol/L pH 7.4 PBS washing solution; Dissolve 2.9 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 0.295 g of sodium dihydrogen phosphate, 8.5 g of sodium chloride and 5 ml of polysorbate 80 in water and dilute to 1000 ml.

(4) Substrate buffer solution: Dissolve 12.9 g of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 3.26 g of citric acid in water and dilute to 700 ml.

(5) Substrate solution: Dissolve 4 mg of o-phenylenediamine in 10 ml of substrate buffer solution and add 4 μl of 30% hydrogen peroxide.

(6) Stopping solution: 1 mol/L sulfuric acid solution.

Preparation of test sample

The test samples include hybridoma cell strain, ascites and final bulk or final product of monoclonal antibody. Hybridoma cell strain shall be subject to in vitro infectivity assay, animal antibody production test and infectivity assay in embryonated eggs; however, ascites and final bulk or final product of monoclonal antibody shall be subject to animal antibody production test and infectivity assay in embryonated eggs.

(1) Test sample for in vitro infectivity assay: Three flasks of hybridoma cells growing well are subject to freezing and thawing at -40°C for 3 times, pooled aseptically and dispensed into small tubes, 3 ml per tube. Seal the tubes with rubber stoppers and store at -40°C .

(2) Test sample for animal antibody production test: Ascites and final bulk or final product of monoclonal antibody need no treatment and shall be stored at -20°C . However, hybridoma cells shall be treated by the following method before use.

Remove the media in seven flasks in which hybridoma cells grown well. Wash the cells down with PBS by blowing slightly and transfer into small tubes. Wash the flask once with PBS to collect the residual cells into the same tube and centrifuge at 1000 r/min for 10 minutes. Discard the supernatant and resuspend the cells with PBS. Repeat the above procedure twice. Resuspend the pooled cell pellet in 4 ml of PBS. After 3 times of freezing and thawing, ultrasonicate the cell suspension and centrifuge at 10000 r/min for 30 minutes. Collect the supernatant and centrifuge at 40000 r/min for 4 hours. Discard the supernatant and dissolve the pellet in a quantity of PBS to obtain the antigen for animal antibody production test. Store the antigen at -40°C .

Procedure

The methods for detection of murine viruses include in vitro infectivity assay, animal antibody production test, infectivity test in embryonated eggs, and so on.

1. In vitro infectivity assay: Detect the presence of unknown virus antigen in test sample with known virus antibody.

(1) Cell culture: Select the cells sensitive to the virus to be tested. Six flasks of each kind of cells growing well shall be used. Wash the cells twice with 0.01 mol/L pH 7.4 PBS. Inoculate 0.3 ml of test sample of each batch into each of four flasks, and 0.3 ml of PBS into the other two flasks as

negative control. After adsorption at 37°C for one hour, discard the adsorbed test sample fluid and add a quantity of cell maintenance media into each flask. Observe the morphology of cells every day under microscope and record the results. Change the media every 3-4 days. Maintain the first passage of cells for 10-14 days. After 3 times of freezing and thawing, pool the two flasks of negative control and four flasks of test sample separately. Inoculate the harvested cell suspensions in test and control groups into the same kind of cells, and change the medium every 3-4 days.

(2) Smear: After incubation of the pooled cell suspension for 10-14 days, remove the maintenance media and wash the cells twice with PBS. Add 0.15 ml of digestion solution into each flask to detach the cells. Pipette the cell suspension into a tube and wash twice with PBS. Resuspend the cell pellet with a quantity of PBS. Smear the normal cells in negative control group onto the first row of wells on a glass slide, and the test sample onto the second row. Dry the slide by blowing and fix the cells with acetone to obtain the cell slide of test sample. Store the slide at -40°C .

Indirect immunofluorescence assay: Prepare a slide of known virus antigen, make the known specific positive and negative sera 5- to 20- fold dilutions with PBS respectively. Examine the slide of test sample using the prepared slide of known virus antigen as a serum control. Add the known positive and negative sera diluted 10-fold with PBS respectively into different wells on the slide of test sample and incubate at 37°C for 30 minutes in a wet box. Wash the slide 3 times, each by immersing with PBS for 5 minutes, and dry. Drop fluorescein-labelled antibody into each well and incubate at 37°C for 30 minutes. Wash the slides 3 times each by immersing with PBS for 5 minutes, then wash once with water and dry. Add 50% glycerin onto the slides and cover with a cover glass. Examine the slide under microscope.

(3) Result evaluation

The test is valid if on the slides of known virus antigen, negative control sera show no fluorescence with normal cell and virus cell wells, while positive control sera show no fluorescence with normal cell wells but show fluorescence with virus cell wells; and if on the slides of test sample, negative control sera show no fluorescence with normal cell and test sample wells, while positive control sera show no fluorescence with normal cell well.

The test is invalid if negative control sera show fluorescence with normal cell and test sample wells, or positive control sera show fluorescence with normal cell well.

The result is judged as positive if, on the slide of test sample, the positive control sera show fluorescence with test sample well.

2. Animal antibody production test

Preparation of antibody to test sample: Inject each

batch of test sample into 50 SPF BALB/c or KM mice according to the requirements in the following table:

Mouse	Number of mouse		Injection Route	Dosage (ml/mouse)	Note
	Test group	Control group			
Suckling	10		i. m.	0.03	Observe the mice for 4 weeks. The survival rate shall be not less than 80%.
Aged 3-4 weeks	10	10	i. p.	0.03	Observe the mice for 4 weeks. The survival rate shall be not less than 80%.
Aged 6-8 weeks	10	10	i. m. and i. p.	i. m. : 0.1 i. p. : 0.2	Inject 2 doses at an interval of 10 days. Bleed the mice 14 days after the second injection. Inject PBS into the mice in control group.

Serological examination: Bleed the mice injected i. m. and i. p. with test sample and PBS (control) and separate sera for detecting antibody by ELISA. Coat a 96-well microtitre plate with virus and normal cell antigens, 0.1 ml per well. Incubate the plate at 37°C for one hour, allow to stand at 4°C overnight. Wash the plate sufficiently with washing solution then pat to dry. Add test sample into one virus antigen well and one normal cell antigen well respectively and incubate at 37°C for one hour. Wash the plate sufficiently with washing solution then pat to dry. Add enzyme-labelled secondary antibody into each well and incubate at 37°C for one hour. Wash the plate sufficiently with washing solution then pat to dry. Add 0.1 ml of substrate solution into each well and incubate at 37°C for 10-20 minutes. Stop the reaction by adding 0.1 ml of 1 mol/L sulfuric acid solution into each well when positive serum control wells develop colour, but negative control wells show no colour. Read the absorbance of each well.

(4) Result evaluation

If the value of P/N is not less than 2.1, the result is judged as positive;

If the value of P/N is 1.5-2.0, the result is judged as doubtful;

If the value of P/N is less than 1.5, the result is judged as negative.

P = Absorbance of conjugate of murine sera immunized with test sample and virus antigen-Absorbance of conjugate of murine sera immunized with test sample and normal cell antigen

N = Absorbance of conjugate of murine sera immunized with control and virus antigen-Absorbance of conjugate of murine sera immunized with control and normal cell antigen

3. Infectivity assay in embryonated eggs

Observe the embryonated eggs 24 hours before inoculation. Clear blood vessels and dark shadow shall be observed in live embryonated eggs, and blastokinesis may be observed in bigger ones. However, if the chick embryo is dead, the blood vessels are dusky and blurred, and no blastokinesis shall be observed. Examine the eggs for vitality of chick embryo again with inspection lamp just before inoculation, and mark the locations of air chamber and embryo. Inoculate aseptically the test sample into yolk sac, allantoic cavity and villous allantoic membrane, incubate the embryonated eggs for 5 days and observe daily. Harvest the yolk sac, villous allantoic membrane and allantoic liquid by an aseptic operation. Grind yolk sac and villous allantoic membrane and centrifuge to collect the supernatant. Carry out hemagglutination tests on the supernatant and allantoic fluid with guinea pig or chick erythrocyte separately.

Take a microtitre plate of eight wells in each row. Add 50 μ l of physiological saline into the 2nd to the 8th well. Add 50 μ l of test sample treated by above-mentioned procedures into the 1st and the 2nd well respectively. Dilute the test sample 2-fold serially by transferring 50 μ l of sample from the 2nd to the 3rd well with a pipette, then 50 μ l from the 3rd well to the 4th well, and so on, until 50 μ l is transferred from the 6th to the 7th well. Pipette out 50 μ l of sample from the 7th well and discard. The 8th well is used as a control. Add 50 μ l of 1% guinea pig erythrocyte suspension into the 1st to the 8th well and mix thoroughly. The test is performed in duplicate. Allow the two microtitre plates stand at 4°C and room temperature respectively until clear negative result is observed in control well.

Result evaluation:

++++; Erythrocytes are evenly dispersed at the bottom of well;

+++; Erythrocytes are evenly dispersed at the bottom of well, but their borders are irregular and show a tendency of decline.

++; Erythrocytes form a small loop at the bottom of well, surrounded by small clots;

+: Erythrocytes form a small block at the bottom of well, bordered with a small number of clots;

—; Erythrocytes are centralized at the bottom of well and form red point with a dense border.

If the hemagglutination reaction of “++” or more appears, the result shall be judged as positive.

Appendix XIII

XIII A Test Requirements for SPF Chicken Embryos

Specific pathogen-free (SPF) chicks represent that the chicks are bred under a strictly controlled surveillance to meet the requirements for specified microbiological examination. SPF chick embryo refers to the embryo which is hatched from a fertilized egg laid by SPF hen and incubated under an appropriate condition for the production of biologics. The quality of the SPF chick embryos shall be controlled through detecting specific pathogenic microorganisms in SPF chick flocks and their eggs.

Detection of pathogens and method

Detection of pathogens for surveillance on SPF chick embryos and the detection methods used are listed as follows:

No.	Pathogenic microorganism	Demand	Method
1	<i>Salmonella pullorum</i>	●	SPA, IA, TA
2	Avian influenza virus (type A)	●	AGP, HI
3	Infectious bronchitis virus	●	AGP, SN, HI
4	Infectious bursal disease virus	●	AGP, SN
5	Infectious laryngotracheitis virus	●	AGP, SN
6	Newcastle disease virus	●	HI
7	Fowl pox virus	●	AGP
8	Marek's disease virus	●	AGP
9	<i>Haemophilus paragallinarum</i>	●	SPA
10	<i>Pasteurella multocida</i>	○	AGP
11	Avian adenovirus Group III	●	HI
12	<i>Mycoplasma gallisepticum</i>	●	SPA, HI
13	<i>Mycoplasma synoviae</i>	●	SPA, HI
14	Avian encephalomyelitis virus	●	AGP, EST, SN
15	Lymphoid leukosis virus	●	ELISA
16	Reticuloendotheliosis virus	●	AGP
17	Avian reovirus	●	AGP
18	Avian adenovirus Group I	●	AGP
19	Chicken infectious anaemia virus	●	IFA

Note: ●=Obligatory, result shall be negative
 ○=In case of need, result shall be negative
 SPA=Serum Plate Agglutination Test
 IA=Isolation of pathogen
 AGP=Agar Diffusion Test
 HI=Haemagglutination Inhibition Test
 ELISA=Enzyme-Linked Immunosorbent Assay
 EST=Embryo Sensitive Test
 SN=Serum Neutralization Test
 TA=Test-tube Agglutination Test
 IFA=Indirect Immunofluorescence Assay

Procedure

(1) Sampling

Fresh egg samples are required for detecting avian encephalomyelitis virus and avian lymphoid leukosis virus. Serum samples are required for detecting other pathogenic microbial infections in chicken. The amount of each sample shall be at least 1 ml. To avoid contamination, sampling shall follow the aseptic procedures.

(2) Amount of egg samples

For detecting lymphoid leukosis virus antigen, 200 eggs shall be sampled from each flock of chickens, one egg from each hen (If the chicken number in one flock is less than 200, sample shall be taken from each individual).

For detecting avian encephalomyelitis virus, at least 50 eggs shall be sampled from each flock, one egg from each hen (If the chicken number in one flock is less than 50, samples shall be taken from each individual).

For detecting other pathogenic microorganism infections, samples shall be taken at random at a rate of 5% from each chicken flock. For the flock consisting of less than 200 chickens, sample shall be taken at a proportion of 10%-15%.

(3) Storage and shipping of the samples

After collection, samples shall be sent to the laboratory for tests as soon as possible.

If samples can not be sent to the laboratory in time, sera shall be stored frozen at or below -15°C. Eggs shall be stored at 4-10°C. The storage period shall not exceed one week.

Samples shall be marked with distinct labels. Delivery sheets shall be attached indicating the names and numbers of chicken groups as well as the names and quantity of the samples.

During shipping of samples, care shall be taken to avoid temperature rising and the breakage of eggs.

Result evaluation

If any detecting result of sample fails to meet the requirements in this Appendix, the sampling batch of chick embryo shall be judged as unqualified.

XIII B Test Requirements of Microbes for Laboratory Animals

The Requirements (quoted from GB14922.1-2001)

are suitable for guinea pig, hamster, rabbit, dog and monkey, as well as the mouse and rat above clean level.

1. Microbiological grade of laboratory animals

(1) Conventional (CV) animals: The CV animals shall be free from pathogens of specified anthro-pozoonosis or fulminating zoonoses.

(2) Clean (CL) animals: Except the pathogens that shall be excluded for the CV animals, the CL animals shall be free from the pathogens seriously harmful to animals or significantly interfering scientific research.

Table 1 Test requirements for pathogens in mouse and rat

Grade of animal		Pathogen	Mouse	Rat
GF	SPF	CL		
		<i>Salmonella spp.</i>	●	●
		<i>Listeria monocytogenes</i>	○	○
		<i>Yersinia pseudotuberculosis</i>	○	○
		<i>Yersinia enterocolitica</i>	○	○
		Pathogenic dermal fungi	○	○
		<i>Streptobacillus moniliformis</i>	○	○
		<i>Bordetella bronchiseptica</i>		●
		<i>Mycoplasma spp.</i>	●	●
		<i>Corynebacterium kutscheri</i>	●	●
		Tyzzar's organism	●	●
		<i>Escherichia coli</i> O115 a, C, K (B)	○	
		<i>Pasteurella pneumotropica</i>	●	●
		<i>Klebsiella pneumoniae</i>	●	●
		<i>Staphylococcus aureus</i>	●	●
		<i>Streptococcus pneumoniae</i>	○	○
		β-Hemolytic streptococcus	○	○
		<i>Pseudomonas aeruginosa</i>	●	●
		No any detectable microorganisms	●	●

● Obligatory, result shall be negative; ○ In case of need, result shall be negative.

Table 2 Test requirements for pathogens in guinea pig, hamster and rabbit

Grade of animal		Pathogen	Guinea pig	Hamster	Rabbit
GF	SPF	CL			
		CV			
		<i>Salmonella spp.</i>	●	●	●
		<i>Listeria monocytogenes</i>	○	○	○
		<i>Yersinia pseudotuberculosis</i>	○	○	○
		<i>Yersinia enterocolitica</i>	○	○	○
		Pathogenic dermal fungi	○	○	○
		<i>Streptobacillus moniliformis</i>	○	○	
		<i>Pasteurella multocida</i>	●	●	●
		<i>Bordetella bronchiseptica</i>	●	●	●
		Tyzzar's organism	●	●	●
		<i>Pasteurella pneumotropica</i>	●	●	●
		<i>Klebsiella pneumoniae</i>	●	●	●
		<i>Staphylococcus aureus</i>	●	●	●
		<i>Streptococcus pneumoniae</i>	○	○	○
		β-hemolytic streptococcus	●	○	○
		<i>Pseudomonas aeruginosa</i>	●	●	●
		No any detectable microorganisms	●	●	●

Note: ● Obligatory, result shall be negative; ○ In case of need, result shall be negative.

(3) Specific pathogen-free (SPF) animals: Except the pathogens that shall be excluded for the CL animals, the SPF animals shall be free from the major potentially infectious pathogens, conditional pathogens or the pathogens significantly interfering scientific experiments.

(4) Germ-free (GF) animal: They shall be free from any detectable organisms.

2. Test requirements

(1) Appearance: The animals shall be healthy and show no abnormal signs in appearance.

(2) Pathogens: See Tables 1, 2 and 3.

(3) Viruses: See Tables 4, 5 and 6.

Table 3 Test requirements for pathogens in dog and monkey

Grade of animal		Pathogen	Dog	Monkey
SPF	CV	<i>Salmonella spp.</i>	●	●
		Pathogenic dermal fungi	●	●
		<i>Brucella spp.</i>	●	
		<i>Leptospira spp.</i>	△	
		<i>Shigella spp.</i>		●
		<i>Mycobacterium tuberculosis</i>		●
		<i>Leptospira spp.</i> *	●	
		<i>Yersinia enterocolitica</i>	○	○
		<i>Campylobacter jejuni</i>	○	○

Note: ● Obligatory, result shall be negative; ○ In case of need, result shall be negative; △ In case of need, immunization is permitted; * Immunization is not permitted, and the result shall be negative.

Table 4 Test requirements for viruses in mouse and rat

Grade of animal		Virus	Mouse	Rat
GF	SPF	CL		
		Lymphocytic choriomeningitis virus (LCMV)	○	
		Hantavirus (HV)	○	●
		Ectromelia virus (Ect.)	●	
		Mouse hepatitis virus (MHV)	●	
		Sendai virus (SV)	●	●
		Pneumonia virus of mice (PVM)	●	●
		Reovirus type III (Reo-3)	●	●
		Minute virus of mice (MVM)	●	
		Theiler's mouse encephalomyelitis virus (TMEV)	○	
		Mouse adenovirus (Mad)	○	
		Polyoma virus (POLY)	○	
		Rat parvovirus (KRV)		●
		Rat parvovirus (H-1)		●
		Rat coronavirus (RCV)/Sialodacryoadenitis virus (SDAV)		●
		No any detectable virus.	●	●

Note: ● Obligatory, result shall be negative; ○ In case of need, result shall be negative.

Table 5 Test requirements for viruses in guinea pig, hamster and rabbit

Grade of animal				Virus	Guinea pig	Hamster	Rabbit
GF	SPF	CL	CV	Lymphocytic choriomeningitis virus (LCMV)	●	●	
				Rabbit hemorrhagic disease virus (RHDV)	▲		
				Sendai virus (SV)	●	●	
				* Rabbit hemorrhagic disease virus (RHDV)			●
				Sendai virus (SV)			●
				Pneumonia virus of mice (PVM)	●	●	
				Reovirus type III (Reo-3)	●	●	
				Rotavirus (RRV)			●
				No any detectable virus	●	●	●
				Note: ● Obligatory, result shall be negative; ▲ Obligatory, immunization is permitted; * Immunization is not permitted, and the result shall be negative.			

Table 6 Test requirements for viruses in dog and monkey

Grade of animal		Virus	Dog	Monkey
SPF	CV	Rabies virus (RV)	▲	
		Canine parvovirus (CPV)	▲	
		Canine distemper virus (CDV)	▲	
		Infectious canine hepatitis virus (ICHV)	▲	
		Cercopithecine herpesvirus Type 1(BV)		●
		Simian retrovirus D (SRV)		●
		Simian immunodeficiency virus (SIV)		●
		Simian T lymphotropic virus Type 1(STLV-1)		●
		Simian pox virus (SPV)		●
		The above 4 kinds of viruses are not permitted to be used for immunization.	●	
Note: ● Obligatory, result shall be negative; ▲ Obligatory, the animals shall be immunized.				

XIII C Test Requirements of Parasites for Laboratory Animals

The Requirements (quoted from GB14922.2-2001) are suitable for hamster, guinea pig, rabbit, dog and monkey, as well as the mouse and rat above clean level.

1. Parasitological grade of laboratory animals

(1) Conventional (CV) animals; They shall be free from the specified parasites causing Anthroponosis.

(2) Clean (CL) animals; Except the parasites that shall be excluded for the CV animals, the CL animals shall be free from the parasites seriously harmful to animals or significantly interfering scientific research.

(3) Specific pathogen-free (SPF) animals; Except

the parasites that shall be excluded for the CL animals, the SPF animals shall be free from the major potentially infectious parasites, conditional pathogenic parasites or the parasites significantly interfering scientific experiments.

(4) Germ-free (GF) animals; They shall be free from any detectable organisms.

2. Test requirements

(1) Appearance; The animals shall be healthy and show no abnormal signs in appearance.

(2) Parasites; See Tables 1, 2 and 3.

Table 1 Test requirements for parasites in mouse and rat

Grade of animal				Parasites	Mouse	Rat
GF	SPF	CL	CV	Ectoparasites	●	●
				<i>Toxoplasma gondii</i>	●	●
				<i>Encephalitozoon cuniculi</i>	○	○
				<i>Pneumocystis carinii</i>	○	○
				All helminths	●	●
				Flagellates	●	●
				Ciliates	●	●
				No any detectable parasites	●	●
				Note: ● Obligatory, result shall be negative; ○ In case of need, result shall be negative.		

Table 2 Test requirements for parasites in guinea pig, hamster and rabbit

Grade of animal				Parasites	Guinea pig	Hamster	Rabbit
GF	SPF	CL	CV	Ectoparasites	●	●	●
				<i>Toxoplasma gondii</i>	●	●	●
				<i>Encephalitozoon cuniculi</i>	○		○
				<i>Eimeria spp.</i>		○	○
				<i>Pneumocystis carinii</i>			●
				All helminths	●	●	●
				Flagellates	●	●	●
				Ciliates	●		
				No any detectable parasites			
				Note: ● Obligatory, result shall be negative; ○ In case of need, result shall be negative.			

Table 3 Test requirements for parasites in dog and monkey

Grade of animal				Parasites	Dog	Monkey
SPF	CV			Ectoparasites	●	●
				<i>Toxoplasma gondii</i>	●	●
				All helminths	●	●
				<i>Entamoeba spp.</i>	○	●
				<i>Plasmodium spp.</i>		●
				Flagellates	●	●
				Note: ● Obligatory, result shall be negative; ○ In case of need, result shall be negative.		

XIII D Test Requirements for Calf Serum

Protein content 3.5%-5.0% (Appendix VI B, method 1).

Sterility test Complies with the test for sterility (Appendix XIII A).

Bacterial endotoxin Not more than 10 EU/ml (Appendix XIII E, the limit test of gel-clot method).

Bovine diarrhea virus The serum shall be free from contamination with bovine diarrhea virus. The virus shall be detected by the following culture method or other suitable method.

Culture method: Prepare primary bovine kidney cells into a suspension with MEM and inoculate with an equal volume of calf serum to be tested. Incubate at 37°C for 5-7 days and observe the CPE. Positive and negative control tests shall be performed in parallel.

Hemoglobin Cyanomethemoglobin method or other suitable method shall apply. The hemoglobin content in calf serum shall be not more than 0.02%.

Coliphage Bacteriophage plaque and proliferation method shall apply. No coliphage shall be detected.

Mycoplasma Culture method and DNA staining method shall apply (Appendix XIII B). No contamination of mycoplasma shall be detected.

Test for proliferation of supporting cells The test is performed by using Sp2/0-Ag14 cell or other suitable continuous cell line which is anchorage-independent.

(1) Determination of cell growth curve: Prepare cell culture medium with the test sample to a concentration of 10%. Inoculate cells into the medium to a final concentration of 1×10^4 cells per ml. Count live cells every day for 7 days and plot a cell growth curve. The maximum proliferation concentration of cells shall be not less than 1×10^6 cells per ml.

(2) Determination of cell doubling time: Calculate the cell doubling time according to the cell growth curve by the following equation;

The cell doubling time = T/A $A = \log_2 Y/X$

Where: T = Time for cell growth, hour;

Y = Cell count one day before the growth reaches the peak value;

X = Number of cells inoculated.

The doubling time of Sp2/0-Ag14 cells shall be not more than 20 hours.

(3) Determination of cloning rate: Dilute the cells by limited dilution method and inoculate onto a 96-well cell culture plate, one cell per well. Incubate the plate at 37°C in a 5% carbon dioxide incubator for one week and calculate the cloning rate by the following equation.

Cloning rate = $(A/B) \times 100\%$

Where: A = Number of wells showing cell growth;

B = Total number of wells inoculated with cells.

Appendix XV Culture Media for Biochemical Reactions of Bacteria and Test Method

The following culture media are commonly used to test the biochemical reactions arisen from bacteria in the metabolism of carbohydrates, amino acids and proteins as well as the utilization of carbon and nitrogen sources.

1. Sugar or alcohol fermentation medium

(1) Formula

Basal medium:	
Peptone	10 g
Sodium chloride	5 g
0.5% Acid fuchsin indicator solution	10 ml
(or 0.4% bromothymol blue solution)	6 ml
Water	1000 ml.
Sugars or alcohols	

0.5 g per 100 ml of basal medium.

(2) Method for preparation

Dissolve peptone and sodium chloride in water by slightly warming. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Add acid fuchsin solution (or bromothymol blue solution) and mix well. To 100 ml of the solution, add one kind of sugar, alcohol or glycoside. Mix well. Dispense the medium into small tubes (if observation of gas production is needed, Durham inverted tubes are placed in small tubes) and sterilize at 116°C for 15 minutes.

Sugars, alcohols or glycosides commonly used: arabinose, xylose, rhamnose, glucose, fructose, mannose, galactose, maltose, lactose, sucrose, trehalose, cellubiose, melibiose, raffinose, melezitose, inulin, dextrin, starch, mannitol, dulcitol, sorbitol, inositol, glycerol, salicin, esculin, etc.

(3) Use

The medium is used to differentiate various kinds of bacteria through the biochemical reactions of sugar fermentation. Fermentation may produce acid and change the colour of medium (The colour of medium containing acid fuchsin indicator may change from colourless to red or even to yellow; the colour of medium containing bromothymol blue indicator may change from blue colour to yellow). When gas is produced, small air bubbles can be seen in Durham inverted tube.

2. Esculin Medium

(1) Formula

Peptone	5 g
Dipotassium hydrogen phosphate	1 g
Ferric citrate	0.5 g
Esculin	3 g

Water 1000 ml

(2) Method for preparation

Mix the above ingredients except esculin and dissolve the solids by slightly warming. Add esculin and mix well. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Dispense the medium into test tubes and sterilize at 121°C for 15 minutes.

(3) Use

The medium is used to differentiate bacteria based on the test of esculin hydrolysis. The production of a brownish black precipitate indicates a positive reaction.

3. Phosphate glucose peptone water medium

(1) Formula

Peptone	7 g
Dipotassium hydrogen phosphate	3.8 g
Glucose	5 g
Water	1000 ml

(2) Method of preparation

Mix the above ingredients and dissolve the solids by slightly warming. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Dispense the medium into small test tubes and sterilize at 121°C for 15 minutes.

(3) Use

The medium is used to differentiate bacteria through methyl red test (M-R reaction) and acetylmethylcarbinol test (V-P reaction).

① Methyl red test (M-R reaction): Inoculate suspected colony or culture on slant into phosphate glucose peptone water medium and incubate at an appropriate temperature for 2-5 days. Add several drops of methyl red solution (Dissolve 0.1 g of methyl red in 300 ml of 95% ethanol and dilute to 500 ml with water) into the culture tube and observe immediately. The appearance of bright red or orange red colour indicates a positive reaction and yellow colour indicates a negative reaction.

② Acetylmethyl-carbinol test (V-P reaction): Inoculate suspected colony or culture on slant into phosphate glucose peptone water medium and incubate at an appropriate temperature for 48 hours. Add 1 ml of α -naphthol ethanol solution (Dissolve 5 g of α -naphthol in absolute ethanol and dilute to 100 ml) into 2 ml of the culture and mix well. Then, add 0.4 ml of 40% potassium hydroxide solution and mix thoroughly. If a red colour appears instantly or within several minutes,

it indicates a positive reaction. If no red colour appears, it indicates a negative reaction. For a negative reaction, observe again after incubation in a 35°C water bath for 4 hours.

4. Peptone water medium

(1) Formula

Peptone	10 g
Sodium chloride	5 g
Water	1000 ml

(2) Method of preparation

Mix the above ingredients and dissolve the solids by slightly warming. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Dispense the medium into small test tubes and sterilize at 121°C for 15 minutes.

(3) Use

The medium is used to differentiate bacteria based on their ability to decompose tryptophan and produce indole.

Indole test: Inoculate the suspected colony or culture on agar slant into peptone water medium and incubate at 35°C for 24-48 hours, or 4-5 days if necessary. Add several drops of indole test solution along the wall of test tube. A positive test shows a rosered colour on the surface of the liquid and a negative test shows no colour change.

Indole test solution: Dissolve 5 g of p-dimethylaminobenzaldehyde in 75 ml of pentanol (or iso-pentanol) and shake sufficiently. After the dissolution is completed, add slowly 25 ml of concentrated hydrochloric acid dropwise with constant shaking to prevent the colour from becoming dark due to the sudden increase of temperature. Alternatively, add 1 g of p-dimethylaminobenzaldehyde into 95 ml of 95% ethanol and shake sufficiently to dissolve completely. Add slowly 20 ml of concentrated hydrochloric acid dropwise.

5. Triple sugar iron agar medium

(1) Formula

Peptone	20 g
Beef extract	5 g
Lactose	10 g
Sucrose	10 g
Glucose	1 g
Sodium chloride	5 g
Ferrous sulfate	0.2 g
Sodium thiosulfate	0.2 g
0.2% Phenol red solution	12.5 ml
Agar	12-15 g
Water	1000 ml

(2) Method of preparation

Mix the above ingredients except lactose, sucrose, glucose, phenol red solution and agar and dissolve by heating. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Add agar and swell by heating. Then, add the rest ingredients and mix well. Dispense the medium and sterilize at 121°C for 15 minutes, allow to stand to form a short slant with high bottom (2-3 cm).

(3) Use

The medium is used for the preliminary identification of bacteria in Enterobacteriaceae based on the fermentation of sugars and hydrogen sulfide production.

Test method and result observation: Streak the suspected colony or culture from slant onto the medium slant and also puncture into the deep bottom. Incubate at 35°C for 24-48 hours and observe the result. When the colour at the bottom of the medium becomes yellow, it indicates a positive reaction of glucose fermentation. When the colour of the slant becomes yellow, it indicates a positive reaction of lactose and sucrose fermentation. When a black colour appears at the bottom or in the whole medium, it indicates the production of hydrogen sulfide.

6. Kligler double sugar iron agar medium

(1) Formula

Peptone	20 g
Beef extract	3 g
Yeast extract	3 g
Lactose	10 g
Glucose	1 g
Sodium chloride	5 g
Ferric citrate	0.3 g
Sodium thiosulfate	0.3 g
0.2% Phenol red solution	12.5 ml
Agar	12-15 g
Water	1000 ml

(2) Method for preparation

Mix the above ingredients except lactose, phenol red solution and agar and dissolve the solids by heating. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Add agar and swell by heating. Add the rest ingredients and mix well. Dispense the medium and sterilize at 121°C for 15 minutes, allow to stand to form a short slant with high bottom (2-3 cm).

(3) Use

The medium is used for the preliminary identification of bacteria in Enterobacteriaceae by test of sugar fermentation and hydrogen sulfide production.

Test method and result observation: Streak the suspected colony or culture from slant onto the medium slant and also puncture into the deep bottom. Incubate at 35°C for 24-48 hours and observe the result. When the colour at the bottom of the medium becomes yellow, it indicates a positive reaction of glucose fermentation. When the colour of the slant becomes yellow, it indicates a positive reaction of lactose fermentation. When the colour of slant becomes red, it indicates a negative reaction of lactose fermentation. When a black colour appears at the bottom or in the whole medium, it indicates the production of hydrogen sulfide.

7. Urea medium

(1) Formula

Peptone	1 g
Glucose	1 g
Sodium chloride	5 g

Dipotassium hydrogen phosphate	2 g
0.2% Phenol red solution	6 ml
20% Sterile urea solution	100 ml
Water	1000 ml

(2) Method for preparation

Mix the above ingredients well except urea solution. Adjust the pH so that after sterilization it is 6.9 ± 0.1 . Sterilize the medium at 121°C for 15 minutes. Cool down to $50-55^\circ\text{C}$. Add in sterile urea solution (sterilized by membrane filtration) and mix well. Dispense the medium into sterile test tubes.

(3) Use

The medium is used to differentiate the bacteria by the test of urease production.

Test method and result observation; Inoculate the suspected colony or a small amount of culture from slant onto the urea medium. Incubate at an appropriate temperature for 24 hours and observe the result. When the colour of the medium becomes red, it indicates a positive reaction of urease production. If no colour changes, it indicates a negative reaction. The culture with negative result shall be further observed for one week.

8. Phenylalanine agar medium

(1) Formula

Disodium hydrogen phosphate	1 g
Yeast extracts	3 g
DL-Phenylalanine (or L-phenylalanine 1 g)	2 g
Sodium chloride	5 g
Agar	12-15 g
Water	1000 ml

(2) Method for preparation

Dissolve the above ingredients in water except agar. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Add agar and swell by heating. Dispense the medium into test tubes and sterilize at 121°C for 15 minutes, allow to stand to form long slants.

(3) Use

The medium is used to identify bacteria by the test of phenylalanine deaminase (or phenyl pyruvic acid test).

Test method and result observation; Inoculate a large amount of culture from slant onto the phenylalanine agar slant. Incubate at an appropriate temperature for 4 or 18-24 hours. Add 4-5 drops of 10% ferric chloride solution on top of the slant and let them flow down along the slant. The appearance of dark green colour indicates a positive reaction of phenylalanine deaminase. If no colour changes, it indicates a negative reaction.

9. Amino acid decarboxylase test medium

(1) Formula

① Basal medium

Peptone	5 g
Yeast extract	3 g
Glucose	1 g
1.6% Bromocresol purple solution	1 ml

Water	1000 ml
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② Amino acids

L-lysine 0.5 g (Dissolve in alkaline solution)

L-Ornithine 0.5 g (Dissolve in alkaline solution)

L-Arginine 0.5 g (Dissolve in water without adding alkaline solution)

(2) Method for preparation

Prepare the basal medium for use. Add the three kinds of amino acids after dissolution into each 100 ml of the basal medium separately (The final concentration of amino acid is 0.5%). Adjust the pH so that after sterilization it is 6.8. Dispense the medium into small test tubes, 2.5 ml for each, and add liquid paraffin dropwise onto the medium to form a layer. At the same time, dispense a portion of the basal medium into small test tubes as control medium. Sterilize all of media at 116°C for 10 minutes.

(3) Use

The medium is used to differentiate the bacteria by the test of decarboxylase and dihydrolase.

Test method and result observation; Inoculate the suspected culture from slant into the three kinds of media and basal media separately. Incubate at an appropriate temperature for 24-48 hours.

Both the media to be tested and control media shall show yellow colour at the beginning due to glucose fermentation and acid production of bacteria to be tested. If the media to be tested show a purple or purple-red colour during continuous incubation, it indicates a positive reaction. If both the media to be tested and control media still show yellow colour at the end of incubation, the reaction shall be judged as negative.

10. Gelatin medium

(1) Formula

Peptone	5 g
Beef extract	3 g
Gelatin	120 g
Water	1000 ml

(2) Method for preparation

Mix the above ingredients in water and soak for about 20 minutes. Dissolve the solids by heating and adjust the pH so that after sterilization it is 7.3 ± 0.1 . Dispense the medium into small test tubes and sterilize at 121°C for 15 minutes.

(3) Use

The medium is used to differentiate the bacteria by the test of gelatin liquefaction.

Test method and result observation; Inoculate by puncturing a small amount of suspected culture from slant into the gelatine medium and incubate at an appropriate temperature for 24 hours. Take the medium out of the incubator and put into a refrigerator for 10-20 minutes. If the medium is still in liquid form, the test is positive. If the medium solidifies again, the test is negative. Sometimes, the reaction of gelatin liquefaction by bacteria is very slowly. If the liquefaction of gelatin is not seen, the final judgment of a negative reaction shall be made after 1-2 weeks of

continuous incubation.

11. Sodium malonate medium

(1) Formula

Yeast extract	1 g
Sodium chloride	2 g
Glucose	0.25 g
Ammonium sulfate	2 g
Dipotassium hydrogen phosphate	0.6 g
Potassium dihydrogen phosphate	0.4 g
Sodium malonate	3 g
0.4% Bromothymol blue solution	6 ml
Water	1000 ml

(2) Method for preparation

Dissolve the above ingredients in water except bromothymol blue solution. Adjust the pH so that after sterilization it is 6.8. Add the bromothymol blue solution. Dispense the medium into test tubes and sterilize at 121°C for 15 minutes.

(3) Use

The medium is used to differentiate the bacteria by judging whether the bacteria can utilize sodium malonate as a source of carbon for growth and propagation.

Test method and result observation: Inoculate the culture from slant or broth medium into the sodium malonate medium and incubate at an appropriate temperature for 48 hours. Observe the result 24 and 48 hours later respectively. The colour change of the medium from green to blue indicates a positive reaction. If no colour changes or the colour changes from green to yellow, it indicates a negative reaction.

12. Citrate medium

(1) Formula

Sodium chloride	5 g
Magnesium sulfate	0.2 g
Dipotassium hydrogen phosphate	1 g
Ammonium dihydrogen phosphate	1 g
Anhydrous sodium citrate	2 g
1.0% Bromothymol blue solution	10 ml
Agar	14 g
Water	1000 ml

(2) Method for preparation

Mix the above ingredients except bromothymol blue solution and agar and dissolve by slightly warming. Adjust the pH so that after sterilization it is 6.9 ± 0.1 . Add agar and swell by heating. Add the indicator solution and mix well. Dispense the medium into small test tubes and sterilize at 121°C for 15 minutes, allow to stand to form slants.

(3) Use

The medium is used to differentiate bacteria by judging whether the bacteria can utilize citrate as a source of carbon for growth and propagation.

Test method and result observation: Inoculate the suspected colony or culture on slant into the citrate slant and incubate for 48-72 hours. The growth of colony on the slant and the colour change of the medium from green to blue indicate a positive reaction. If no growth of colony is found and the

colour of medium is still green, it indicates a negative reaction. The culture with negative result shall be further incubated and observed until the 7th day.

13. Nitrate peptone water medium

(1) Formula

Peptone	10 g
Yeast extract	3 g
Potassium nitrate	2 g
Water	1000 ml

(2) Method for preparation

Mix the above ingredients and dissolve in water by heating. Adjust the pH so that after sterilization it is 7.3 ± 0.1 . Dispense the medium into small test tubes and sterilize at 121°C for 15 minutes.

(3) Use

The medium is used to differentiate bacteria by judging whether the bacteria can reduce nitrate to nitrite.

Test method and result observation: Inoculate the culture to be tested into nitrate peptone water medium and incubate at an appropriate temperature for 24 hours. Mix the following two solutions (A and B) at equal volumes. To each culture, add 0.1 ml of the mixture. If a red colour appears, it indicates a positive reaction. If no red colour appears, it indicates a negative reaction.

Solution A: Dissolve 5 g of α -naphthylamine in 1000 ml of 5 mol/L acetic acid.

Solution B: Dissolve 8 g of sulfanilic acid (p-aminobenzene sulfonic acid) in 1000 ml of 5 mol/L acetic acid.

14. Litmus milk medium

(1) Formula

Skimmed milk	10 g
10% Litmus solution	0.65 ml
Water	100 ml

(2) Method for preparation

Dissolve the skimmed milk in water and add in 10% litmus solution. Dispense the medium into small test tubes and sterilize at 116°C for 10 minutes.

(3) Use

The medium is used to examine the bacteria for their solidification and fermentation effects on milk.

15. Semisolid agar medium

(1) Formula

Peptone	10 g
Beef extract	3 g
Sodium chloride	5 g
Agar	4 g
Water	1000 ml

(2) Method for preparation

Mix the above ingredients except agar and dissolve the solids by slightly warming. Adjust the pH so that after sterilization it is 7.2 ± 0.2 . Add agar and swell by heating. Dispense the medium into small test tubes and sterilize at 121°C for 15 minutes and solidify by standing vertically.

(3) Use

The medium is used to observe the motility of bacteria as well as to preserve bacterial strains. Test for the mobility of bacteria; Inoculate by puncturing the suspected culture from slant into semisolid medium and incubate at an appropriate

temperature for 24 hours. If the growth of bacteria diffuses around the puncture line, the result is positive. Otherwise, it is negative and the culture shall be further incubated and observed for 2-3 days.

Appendix XV Sterilization

Sterilization is the process to inactivate or remove any viable microorganisms of a product by physical or chemical means. The methods described in this chapter may be used for the sterilization of preparations, raw materials, excipients and medical devices.

Sterility is the absence of any viable microorganisms. Absolute sterility of a product can not be guaranteed nor can it be demonstrated by testing to any batch of sterilized product. In practice, a product is sterilized to decrease the survival probability of microorganisms to a specified level, and is designated as sterility assurance level (SAL). The survival probability of microorganisms of a product treated by terminal sterilization is not more than 10^{-6} . The SAL of a process for a given product is established by appropriate validation studies.

The sterility assurance of a sterile product can not be guaranteed by testing, but depends on the use of the validated sterilization process, good manufacturing practice (GMP), and good quality assurance system. It is essential that the following factors are fully considered in choosing a suitable sterilization procedure, including nature of the product to be sterilized, effectiveness and economy of the procedure, and integrity and stability of the product after sterilization.

It is essential to validate the process before being applied to practice. The validated items include:

- (1) Establish the validation protocols and the evaluation standard.
- (2) Ensure that the equipment are suitable and running effectively.
- (3) Demonstrate that the key equipment and instrumentation are capable of operating within the prescribed parameter criteria.
- (4) Perform replicate cycles by employing actual or simulated product and demonstrate the effectiveness of the process.
- (5) Summarize the records and complete the documents above, to form a validation report.

In manufacture practice, the process of sterilization should be monitored, and the key parameters of the procedure (such as temperature, pressure, duration, humidity, concentration of the gas and the dose of radiation etc.) shall be within the validated limits. The Validation of sterilization process should be repeated at a suitable interval. Revalidation is carried out whenever major changes in the procedure,

including changes in the load, take place.

The sterility assurance is related with the degree of microbial contamination of the product before sterilization and the resistance of the contaminating microorganisms. Therefore, it is essential to control the level of viable microorganisms contamination of a product prior to sterilization and the resistance of the contaminating microorganisms, to choose adequate precautions to minimize the contamination within the prescribed limits in the manufacturing process.

It is necessary to avoid recontamination of the product after sterilization. In all cases, the container and closure are required to maintain the sterility of the product throughout its shelf-life.

Methods of sterilization

The frequently-used methods include steam, dry heat, ionization radiation, gas and filtration. One or combinations of the above methods may be used, depending on the nature of the product. Wherever possible, the terminal sterilization should be chosen. If terminal sterilization is not possible, filtration or aseptic processing is used. Wherever possible, appropriate additional treatment (for example, steam) of a non-final product is applied.

1. Steam sterilization

In this method, the products are placed in a chamber, and sterilized by saturated steam under pressure or overheated water flow which leads to denaturation of protein and nucleic acid to achieve the inactivation of microorganisms. Steam is the most effective and most widely used method, and may be used for pharmaceutical preparations, containers, culture media, sterile coats, plastic plugs, and other materials resistant to high temperature and moisture. Steam sterilization does not guarantee the complete inactivation of bacterial spores, and is often used as an complementary sterile technique for thermo-labile products.

The process is usually carried out by the following conditions:

121°C	15 min
121°C	30 min
116°C	40 min

Other combinations of time and temperature may be used provided that they could give an SAL of 10^{-6} or less. For thermo-stable product, deep sterilization is used, the SAL shall be less than

10^{-12} . For thermolabile products, the standard time of sterilization (F_0) (means the standard duration in the condition of 121.1°C of sterile temperature, one minute of D-value, and 10.0°C of Z-value) usually is not less than eight minutes. If F_0 is less than eight, the contamination of microorganisms should be monitored in the whole manufacturing process, all the measures should be taken to minimize the level of contamination of microorganisms and to ensure that products being sterilized meet the requirements of sterility assurance.

For steam sterilization, the products to be sterilized should be loaded in the sterilizing chamber perfectly, no tightness, to ensure that they are all effectively and equally sterilized.

Knowledge of the coolest part of the chamber is obtained before this method is applied. Put the biological indicator at the coolest part to ensure SAL of the products has been achieved. Spores of *Bacillus stearothermophilus* are usually used as the indicator for this method.

2. Dry heat sterilization

Dry heat sterilization is carried out in an oven or tunnel equipment for sterilization with forced air circulation, where microorganisms and pyrogens are inactivated by high temperature. This method is suitable for products where steam sterilization is inappropriate, such as glass utensils, metal containers, fibre products, solid drugs, and liquid paraffin wax.

The process is usually carried out by heating the product at 160-170°C for 120 minutes or longer, 170-180°C for 60 minutes or longer, or 250°C for 45 minutes or longer. Other combinations of time and temperature may be used provided that they could give an SAL of 10^{-6} or less. For thermo-stable product, sterilize it until the SAL is $\leq 10^{-12}$ wherever possible. In the latter case, sterility test for these products prior to sterilization is not necessary. Dry heat at 250°C for 45 minutes can also remove the pyrogens of containers and other equipment.

Put the products in suitable containers to ensure that they are all effectively and equally sterilized. Knowledge of the coolest part of the chamber is obtained before this method is applied. Spores of *Bacillus subtilis* are usually used as the indicator for this method. Put the biological indicator at the coolest part to ensure SAL of the products has been achieved. Endotoxin inactivation test can verify the effectiveness of depyrogenation. Generally not less than 1000 EU of endotoxin which is derived from *Escherichia coli* may be added into the items prior to depyrogenation, in this case the reduction of the endotoxin is at least 3-log.

3. Ionising radiation sterilization

Sterilization by this method is achieved by exposure of the product to ionizing radiation in the form of gamma radiation from a suitable

radioisotopic source (such as cobalt 60) or of a beam of electrons energized by a suitable electron accelerator. This method may be used for medical devices, containers, manufacturing equipment, and other raw materials and preparations resistant to radiation.

The SAL of the product is not more than 10^{-6} after ionising radiation sterilization. The key parameter of ionising radiation sterilization is mainly absorbed radiation dose. The radiation dose absorbed by the material being irradiated shall be identified according to the suitability of the products and the maximum amount of the contaminated microorganisms and the maximum resistance to radiation. It should be validated before the performance that the safety, efficiency and stability of a product shall not be changed by the radiation dose. A reference absorbed dose is 25 kGy. Use the lowest possible radiation dose for the final product, raw materials and medical devices. Before irradiation, test the number of contaminated microorganisms and the resistance to the irradiation of the product to evaluate the SAL of the procedure.

During the procedure, the radiation absorbed by the product should be monitored regularly by means of suitable chemical or physical methods to ensure that the dose is appropriate. If dosimeters radiated along with sterilized products are adopted, place them at specific positions. Calibrate against a standard source at suitable intervals.

Spores of *Bacillus pumilus* are usually used as the indicator for this method.

4. Gas sterilization

In this method, microorganisms are inactivated in a high-pressure chamber filled with gas of disinfectors, for example, ethylene oxide, hydrogen peroxide, formaldehyde and ozone (O_3). This method is suitable for the products which are stable in these gases. Inflammability, teratogenesis and residue toxicity of the gases should be considered in the process of sterilization.

The most generally used gas in this method which is carried out in a high pressure chamber filled with sterilized gas is ethylene oxide, usually mixed with 80%-90% inert gases. This method is applicable to medical devices and plastic products that are not durable by using high temperature methods. This method is not applicable to products which contain chlorine or can absorb ethylene oxide.

Temperature, humidity and gas concentration in the chamber, as well as the duration could affect the effectiveness of sterilization by using ethylene oxide. The following conditions are recommended;

Temperature; 54°C \pm 10°C.

Pressure; 8×10^5 Pa

Relative humidity; (60 \pm 10)%

Duration; 90 minutes

The process of sterilization is validated before application. During the procedure, the chamber is

vacuumized first, then filled with vapor to the specified humidity and temperature. Fill in the filtered and preheated ethylene oxide. During the process, seriously monitor the temperature, humidity, pressure, concentration of ethylene oxide and time. Use biological indicators to monitor the effectiveness of sterilization, if necessary. The process should be supervised by skilled technicians. After the material to be sterilized is exposed either to ethylene oxide or to a mixture of ethylene oxide with a suitable inert gas, adequate time should be left to allow dispersal of residual ethylene oxide and other volatile residues. It should be monitored that the residues are within the prescribed limits. Eliminate the remaining gas in the product to a degree free of toxicity to human.

Leaking test should be carried out to ensure obturation of the chamber. Package materials and alignment of the products can affect the gas penetration and diffusion. Spores of *Bacillus subtilis* are usually used as the indicator for this method.

5. Filtration

In this method, microorganisms in the gas or liquid products are removed by filtering through a certain type of filter material. It is usually used for thermolabile solutions of drugs and raw materials. Sterilization filter units use microporous filter membranes (hydrophilic or lipophilic) as the filter material. The pore size is usually less than $0.22\ \mu\text{m}$. Avoid loss of solute by absorption on to the filter and to avoid the release of contaminants from the filter. Avoid use of membranes containing asbestos. The filter unit and membranes are sterilized before use. The filter units are cleaned first and then new membranes are used for a new batch of products.

The sterility assurance level in this method is related with bioburden of a product prior to sterilization and the log reduction value (LRV) of the filter unit. Calculate LRV with the following expression:

$$\text{LRV} = \lg N_0 - \lg N$$

Where N_0 represents the number of microorganisms before filtration, and N is the number after filtration.

LRV is used to express the efficiency of filtration. The LRV per cm^2 of active filter surface is not less than 7 for $0.22\ \mu\text{m}$ -pore-size membranes. Two filters may be linked to increase the efficiency.

Key factors, such as pore size, membrane integrity and LRV can not be monitored in the filtration process. Therefore, the integrity and effectiveness of the membranes should be validated before use. Validate the filter unit for at least once in a working day.

The products sterilized by this method should be operated in a cleaned area with rigorous monitoring and a sterile area is recommended. Relative

facility, package container, stopper and other materials should be sterilized by appropriate methods, and recontamination shall be avoided.

Pseudomonas diminuta are usually used as the indicator for this method.

6. Aseptic preparation

Aseptic preparation denotes the manufacturing of sterile preparations under sterile condition. It may include aseptic filling of products into containers and aseptic lyophilization. Filtration is usually used in the latter process.

It is necessary to monitor the environmental of aseptic preparation, and the filtration should be carried out under sterile conditions. The equipment, containers, plugs and other materials should be sterilized by suitable methods and recontamination shall be avoided.

Sterility assurance of this process is carried out by simulating test for sterility by aseptic filling culture media. Sterility of the circumstance, personnel and materials shall be monitored in the whole process.

The aseptic preparation shall be validated at intervals, including periodic environmental filter examination and simulating test for sterility by aseptic filling culture media.

Biological indicators

Biological indicators are preparations of viable microorganisms used to assess the performance of sterilization equipment, to validate and monitor the effectiveness of sterilization process. They usually consist of bacterial spores.

1. The Requirements for microorganisms used as biological indicators

Different biological indicators are used for different sterilization methods. The microorganisms used as biological indicators comply with the following requirements;

- (1) The test strain is more resistant than any possible contaminating microorganisms in the product to be examined.
- (2) The test strain is non-pathogenic.
- (3) The test strain is resistant to mutation and easy to store.
- (4) The test strain is easy to culture. If resting spores are used, they shall be more than 90% of the indicator.

2. Preparation of biological indicators

The biological indicators are prepared using a specified procedure. Characteristics of the microorganisms are determined before use, for example, D-value, which is the duration (expressed in minutes) with which the number of viable organisms are reduce to 10% of the original number at a definite temperature. Use suitable culture media for the incubation. Suspend the cultures, which mainly consist of spores. Store the spores as suspensions in innutrient liquids. Biological indicators consist of a definite number of

one or more types of spores. They are usually placed on an inert carrier, for example a strip of filter paper, a glass slide, stainless or plastic materials. Spore suspensions may be presented in sealed ampoules. Biological indicators are packed with suitable materials, and an expiry date is set. The carriers and package materials protect the biological indicators from any deterioration or contamination, while allowing the sterilizing agent to enter into and contact with the microorganisms. The packages are designed to facilitate storage, transportation, sampling and subculture. Some biological indicators may be inoculated directly into a liquid product to be sterilized or into a liquid product similar to that to be sterilized. In the latter case, the equivalence of the liquid products are demonstrated.

3. Application of biological indicators

In validation of sterilization process, though some parameter can be used, the effectiveness of a sterilization process is most easily assessed by the degree to which biological indicators are inactivated. Commercial biological indicators, or spores prepared from resistant microorganisms isolated from contaminants are used as biological indicators. The resistance, purity and number of spores are validated for the biological indicators. The quantity and resistance of spore of the biological indicators used shall be more or greater than the contamination of the product to ensure the effectiveness of the process. Put the biological indicators at different position of the chamber for the terminal sterilization. Avoid direct contact of the indicator with the product to be sterilized. Incubate the indicators after specified sterilization on suitable culture media to guarantee inactivation of all the spores.

Commercial biological indicators may be used for validation test of deep sterilization. For susceptible products, select suitable strains and spores as the biological indicator and design the process depending on the degree that the products are contaminated. Sterility assurance of these products are assessed by monitoring the number and resistance of contaminating microorganisms and the validation test.

4. Common biological indicators

(1) Steam sterilization Spores of *Bacillus stearothermophilus* (for example, NCTC 10007, NCIMB

8157, ATCC 7953) are recommended as the indicator for steam sterilization. The D-value is 1.5-3.0 minutes, the number of viable spores in each tablet (or ampul) is 5×10^5 - 5×10^6 , and all the microorganisms are inactivated at 121°C for 19 minutes. Spores of *Clostridium sporogenes* (for example, NCTC 8594, NCIMB 8053, ATCC 7955) may also be used, with D-value of 0.4-0.8 minutes.

(2) Dry-heat sterilization Spores of *Bacillus subtilis* (for example, NCIMB 8058, ATCC 9372) are recommended as the indicator for dry-heat sterilization. The D-value is more than 1.5 minutes, the number of viable spores in each tablet is 5×10^5 - 5×10^6 . Use *Escherichia coli* endotoxin for validation of depyrogenation, the amount added is not less than 1000 EU.

(3) Ionising radiation sterilization Spores of *Bacillus pumilus* (for example, NCTC 10327, NCIMB 10692, ATCC 27142) are recommended as the indicator for ionising radiation sterilization. The number of viable spores in each tablet is 10^7 - 10^8 . The D-value is around 3 kGy when a 25 kGy radiation dose is applied. Contaminated microorganisms in the product may be more resistant to radiation than *B. pumilus*. Therefore, the latter can only be used to monitor the process, and cannot be used to establish the radiation dose.

(4) Gas sterilization Spores of *Bacillus subtilis* (for example, NCTC 10073, ATCC 9372) are recommended as the indicator for gas sterilization with ethylene oxide. The number of viable spores in each tablet is 1×10^6 - 5×10^6 . The D-value is more than 2.5 minutes, and the concentration of ethylene oxide is 600 mg/L. The relative humidity is 60%. The microorganisms are inactivated at 54°C for 60 minutes. Spores of *Bacillus stearothermophilus* (for example, NCTC 10007, NCIMB 8157, ATCC 7953) are recommended as the indicator for gas sterilization with hydrogen peroxide.

(5) Filtration *Pseudomonas diminuta* (for example ATCC 19146) is recommended as the indicator when 0.22 µm-pore-size membranes are used for filtration. *Serratia marcescens* (for example, ATCC 14756) is recommended when 0.45 µm-pore-size membranes are used.

Appendix XVI Names, Symbols and Atomic Weights of Elements

C=12.00

Element	Symbol	Atomic weight	Element	Symbol	Atomic weight
Aluminium	Al	26.981538(2)	Magnesium	Mg	24.3050(6)
Antimony (Stibium)	Sb	121.760(1)	Manganese	Mn	54.938049(9)
Argon	Ar	39.948(1)	Mercury (Hydrargyrum)	Hg	200.59(2)
Arsenic	As	74.92160(2)	Molybdenum	Mo	95.94(2)
Barium	Ba	137.327(7)	Nickel	Ni	58.6934(2)
Bismuth	Bi	208.98038(2)	Nitrogen	N	14.0067(2)
Boron	B	10.811(7)	Oxygen	O	15.9994(3)
Bromine	Br	79.904(1)	Palladium	Pd	106.42(1)
Cadmium	Cd	112.411(8)	Phosphorus	P	30.973761(2)
Calcium	Ca	40.078(4)	Platinum	Pt	195.078(2)
Carbon	C	12.0107(8)	Potassium (Kalium)	K	39.0983(1)
Cerium	Ce	140.116(1)	Selenium	Se	78.96(3)
Chlorine	Cl	35.453(2)	Silicon	Si	28.0855(3)
Chromium	Cr	51.9961(6)	Silver (Argentum)	Ag	107.8682(2)
Cobalt	Co	58.933200(9)	Sodium (Natrium)	Na	22.989770(2)
Copper (Cuprum)	Cu	63.546(3)	Strontium	Sr	87.62(1)
Fluorine	F	18.9984032(5)	Sulfur	S	32.065(5)
Gallium	Ga	69.723(1)	Technetium	Tc	[99]
Germanium	Ge	72.64(1)	Tellurium	Te	127.60(3)
Gold (Aurum)	Au	196.96655(2)	Thorium	Th	232.0381(1)
Helium	He	4.002602(2)	Tin (Stannum)	Sn	118.710(7)
Holmium	Ho	164.93032(2)	Titanium	Ti	47.867(1)
Hydrogen	H	1.00794(7)	Tungsten (Wolfram)	W	183.84(1)
Indium	In	114.818(3)	Uranium	U	238.02891(3)
Iodine	I	126.90447(3)	Vanadium	V	50.9415(1)
Iron (Ferrum)	Fe	55.845(2)	Xenon	Xe	131.293(6)
Lanthanum	La	138.9055(2)	Ytterbium	Yb	173.04(3)
Lead (Plumbum)	Pb	207.2(1)	Zinc	Zn	65.409(4)
Lithium	Li	6.941(2)	Zirconium	Zr	91.224(2)

Notes 1. The last digit of an atomic mass is shown in parentheses.

2. The figure in square brackets is the atomic mass of an isotope which exists with the longest half-life.

INDEX

A

- Agarose Electrophoresis A-30
Agkistrodon acutus Antivenin, Equine 166
Agkistrodon halys Antivenin, Equine 164
 Anthrax Antiserum 172
 Anthrax Vaccine (Live) for Percutaneous Scarification 51
 Anti-human T Lymphocyte Porcine Immunoglobulin 204
 Anti-human T Lymphocyte Rabbit Immunoglobulin 207
 Atomic Absorption Spectrophotometry A-17

B

- BCG Vaccine for Intradermal Injection 57
 Biological Activity Test for Recombinant Bovine Basic Fibroblast Growth Factor A-91
 Biological Activity Test for Recombinant Epidermal Growth Factor A-92
 Biological Activity Test for Recombinant Human Granulocyte Colony-stimulating Factor A-89
 Biological Activity Test for Recombinant Human Granulocyte/Macrophage Colony-stimulating Factor A-90
 Biological Activity Test for Recombinant Human Interleukin-2 A-89
 Biological Activity Test for Recombinant Streptokinase A-93
 Biological Activity Test for Interferon A-88
 Botulinum Antitoxins 160
 Botulinum Antitoxins, Freeze-dried 162
 Botulinum Toxin Type A for Injection 211
 Brucellosis Vaccine (Live) for Percutaneous Scarification 54
Bungarus multicinctus Antivenin, Equine 168

C

- Capsules A-10
 Cellulose Acetate Film Electrophoresis A-30
 Chromatography A-21
 Culture Media for Biochemical Reactions of Bacteria and Test Method A-141

D

- Determination of Aluminium Hydroxide (or Aluminium Phosphate) Content A-60
 Determination of Ammonium Sulfate Content A-58
 Determination of Citrate Content A-61
 Determination of Disintegration A-37
 Determination of F (ab)₂ Content in Antitoxin A-68
 Determination of Flocculation Unit of Toxoid A-103
 Determination of Free Formaldehyde Content A-52
 Determination of Free Histamine Phosphate in Human Histamine Immunoglobulin A-48
 Determination of Heparin Content A-84
 Determination of Human Erythrocyte Antibody A-84
 Determination of Human Platelet Antibody A-85
 Determination of IgG Content A-110
 Determination of IgG Monomer and Dimer in Human Immunoglobulins A-55
 Determination of Loss on Drying A-63
 Determination of Metacresol Content A-53
 Determination of Moisture Content A-58
 Determination of Molecular Size for Group A Meningococcal Polysaccharide A-68
 Determination of Molecular Size for Typhoid Vi Polysaccharide A-69

- Determination of Nitrogen A-44
 Determination of O-Acetyl Content A-48
 Determination of Osmolality A-42
 Determination of Particle Size A-41
 Determination of Phenol Content A-53
 Determination of Phosphorus Content A-57
 Determination of pH Value A-35
 Determination of Polymer Content in Human Albumin A-55
 Determination of Potassium Content A-62
 Determination of Prekallikrein Activator Content A-76
 Determination of Protein Content A-45
 Determination of Residual Aluminium Content in Human Albumin A-63
 Determination of Residual Antibiotics A-71
 Determination of Residual Ethanol Content A-47
 Determination of Residual Extraneous DNA A-71
 Determination of Residual Glutaraldehyde Content A-50
 Determination of Residual Host Bacterial Protein (*E. coli*) A-73
 Determination of Residual Host Bacterial Protein (*Pseudomonas*) A-74
 Determination of Residual Host Yeast Protein A-75
 Determination of Residual Murine IgG A-81
 Determination of Residual Polyethylene Glycol Content A-49
 Determination of Residual Polysorbate 80 Content A-50
 Determination of Saccharides and Sugar Alcohol Content in Human Blood Products A-54
 Determination of Sialic Acid Content A-46
 Determination of Sodium Bisulfite Content A-59
 Determination of Sodium Caprylate Content A-51
 Determination of Sodium Chloride Content A-61
 Determination of Sodium Content A-62
 Determination of Thimerosal Content A-57
 Determination of Total Solid A-64
 Determination of Tributylphosphate Content A-51
 Determination of Trichloromethane Content A-54
 Diphtheria and Pertussis Combined Vaccine, Adsorbed 60
 Diphtheria and Tetanus Combined Vaccine, Adsorbed 80
 Diphtheria and Tetanus Combined Vaccine for Adults and Adolescents, Adsorbed 82
 Diphtheria Antitoxin 150
 Diphtheria Antitoxin, Freeze-dried 151
 Diphtheria, Tetanus and Acellular Pertussis Combined Vaccine, Adsorbed 67
 Diphtheria, Tetanus and Pertussis Combined Vaccine, Adsorbed 62
 Diphtheria Vaccine, Adsorbed 75
 Diphtheria Vaccine for Adults and Adolescents, Adsorbed 78
 Disintegration Test for Suppositories and Vaginal Tablets A-39
 Double Immunodiffusion A-66
 Dysentery Vaccine (Live) of *S. flexneri* and *S. sonnei*, Oral 39

E

- Electrophoresis A-30
 Eye Preparations A-8

F

- Flame Photometry A-20
 Fluorimetry A-19

G

- Gas Chromatography A-26

Gas-gangrene Antitoxin (Mixed) 157
 Gas-gangrene Antitoxin (Mixed), Freeze-dried 159
 Gels A-15
 General Requirements for Preparations A-6
 Granules A-12
 Group A Meningococcal Polysaccharide Vaccine 42

H

Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine, Inactivated 97
 Haemorrhagic Fever with Renal Syndrome (Type I) Vaccine, Inactivated 91
 Haemorrhagic Fever with Renal Syndrome (Type II) Vaccine, Inactivated 94
 Hepatitis A (Live) Vaccine, Freeze-dried 136
 Hepatitis A Vaccine, Live 133
 Hepatitis B Vaccine Made by Recombinant DNA Techniques in CHO Cell 130
 Hepatitis B Vaccine Made by Recombinant DNA Techniques in Yeast 127
 High Performance Liquid Chromatography A-22
 Human Albumin 175
 Human Albumin, Freeze-dried 178
 Human Coagulation Factor VII 199
 Human Fibrinogen 201
 Human Hepatitis B Immunoglobulin 183
 Human Hepatitis B Immunoglobulin, Freeze-dried 185
 Human Immunoglobulin 180
 Human Immunoglobulin for Intravenous Injection 197
 Human Immunoglobulin, Freeze-dried 181
 Human Immunoglobulin (pH 4) for Intravenous Injection 193
 Human Immunoglobulin (pH 4) for Intravenous Injection, Freeze-dried 195
 Human Prothrombin Complex 203
 Human Rabies Immunoglobulin 186
 Human Rabies Immunoglobulin, Freeze-dried 188
 Human Tetanus Immunoglobulin 190
 Human Tetanus Immunoglobulin, Freeze-dried 192

I

Immunoblot A-65
 Immunodot A-65
 Immunoelectrophoresis A-67
 Influenza Vaccine (Whole Virion), Inactivated 124
 Injections A-6
In vitro Test for Relative Potency of Recombinant Hepatitis B Vaccine (Yeast) A-87
In vivo Test for Biological Activity of Recombinant Human Erythropoietin A-87
 Isoelectric Focusing Electrophoresis A-32

J

Japanese Encephalitis Vaccine, Inactivated 84
 Japanese Encephalitis Vaccine, Live 88

L

Leptospira Vaccine 45
 Liquids for External Application A-9

M

Measles and Mumps Combined Vaccine, Live 122
 Measles Vaccine, Live 110
 Microbial Limit Tests A-125
 Mouse Monoclonal Antibody Against Human CD3 Antigen

of T Lymphocyte for Injection 209
 Mumps Vaccine, Live 119

N

Naja naja (atra) Antivenin, Equine 170
 Names, Symbols and Atomic Weights of Elements A-150
 Nasal Preparations A-14

O

Ointments, Emulsions A-10

P

Paper Chromatography A-21
 Peptide Mapping A-67
 Plague Vaccine (Live) for Percutaneous Scarification 48
 Poliomyelitis (Live) Vaccine (Monkey Kidney Cell), Oral 142
 Poliomyelitis Vaccine in Dragee Candy (Human Diploid Cell), Live 138
 Poliomyelitis Vaccine in Dragee Candy (Monkey Kidney Cell), Live 146
 Potency Test for Adsorbed Diphtheria Vaccine A-102
 Potency Test for Adsorbed Tetanus Vaccine A-101
 Potency Test for Anti-human T Lymphocyte Immunoglobulin A-98
 Potency Test for Anti-human T Lymphocyte Immunoglobulin A-99
 Potency Test for Botulinum Antitoxin A-106
 Potency Test for Diphtheria Antibody in Human Immunoglobulin A-96
 Potency Test for Diphtheria Antitoxin A-104
 Potency Test for Gas-gangrene Antitoxin A-105
 Potency Test for Human Coagulation Factor II A-93
 Potency Test for Human Coagulation Factor VII A-94
 Potency Test for Human Coagulation Factor IX A-94
 Potency Test for Human Coagulation Factor X A-95
 Potency Test for Human Coagulation Factor VIII A-96
 Potency Test for Rabies Antiserum A-108
 Potency Test for Rabies Vaccine for Human Use A-101
 Potency Test for Snake Antivenins A-107
 Potency Test for Tetanus Antitoxin A-105
 Powders A-13
 Purified Protein Derivative of BCG (BCG-PPD) 267
 Purified Protein Derivative of Brucellin (BR-PPD) 270
 Purified Protein Derivative of Tuberculin (TB-PPD) 265
 Pyrogen Test A-119

R

Rabies Antiserum 173
 Rabies Vaccine (Hamster Kidney Cell) for Human Use 107
 Rabies Vaccine (Vero Cell) for Human Use 100
 Rabies Vaccine (Vero Cell) for Human Use, Freeze-dried 103
 Recombinant Bovine Basic Fibroblast Growth Factor Eye Drops 258
 Recombinant Bovine Basic Fibroblast Growth Factor for External Use 256
 Recombinant Bovine Basic Fibroblast Growth Factor for External Use, Liquid 254
 Recombinant Human Epidermal Growth Factor Derivative for External Use, Liquid 262
 Recombinant Human Epidermal Growth Factor for External Use 260
 Recombinant Human Erythropoietin for Injection (CHO Cell) 242

Recombinant Human Erythropoietin Injection
(CHO Cell) 245

Recombinant Human Granulocyte Colony-stimulating
Factor Injection 247

Recombinant Human Granulocyte/Macrophage
Colony-stimulating Factor for Injection 249

Recombinant Human Interferon $\alpha 2a$ for Injection 219

Recombinant Human Interferon $\alpha 2a$ for Injection
(Yeast) 224

Recombinant Human Interferon $\alpha 2a$ Injection 221

Recombinant Human Interferon $\alpha 2a$ Vaginal
Suppository 226

Recombinant Human Interferon $\alpha 1b$ Eye Drops 217

Recombinant Human Interferon $\alpha 1b$ for Injection 213

Recombinant Human Interferon $\alpha 2b$ for Injection 228

Recombinant Human Interferon $\alpha 2b$ for Injection
(*P. putida*) 232

Recombinant Human Interferon $\alpha 1b$ Injection 215

Recombinant Human Interferon $\alpha 2b$ Injection 230

Recombinant Human Interferon $\alpha 2b$ Injection
(*P. putida*) 235

Recombinant Human Interferon γ for Injection 237

Recombinant Human Interleukin-2 for Injection 239

Recombinant Streptokinase for Injection 251

Requirements for Bacterial and Viral Strains/Seeds Used for
Production and Quality Control of Biologics 4

Requirements for Defining Batches of Biologics 7

Requirements for Filling and Lyophilization of Biologics 7

Requirements for Packaging of Biologics 9

Requirements for Preparation and Calibration of National
Standard Substances of Biologics 5

Requirements for Preparation and Control of Animal Cell
Substrates Used for Production of Biologics 17

Requirements for Quarantine and Management of Horses
Used for Production of Biologics 11

Requirements for Source Plasma of Blood Products 13

Requirements for Storage and Shipping of Biologics 10

Rubella Vaccine (Human Diploid Cell), Live 113

Rubella Vaccine (Rabbit Kidney Cell), Live 116

S

Schick Test Toxin 273

SDS-Polyacrylamide Gel Electrophoresis A-31

Size Exclusion Chromatography A-27

Spectrophotometry A-16

Sprays A-11

Sterility Test A-113

Sterilization A-146

Suppositories A-7

T

Tablets A-9

Test for Abnormal Toxicity A-124

Test for Activated Coagulation Factor Activity A-83

Test for Adventitious Viruses A-118

Test for Anti-A and Anti-B Hemagglutinins A-78

Test for Anticomplement Activity A-79

Test for Bacterial Endotoxin A-120

Test for Blood Group A-like Substance A-78

Test for Fc Function in Human Immunoglobulin A-97

Test for Human Thrombin Activity A-83

Test for Losing Rate of Plasmid A-77

Test for Minimum Fill A-41

Test for Murine Virus A-134

Test for Mycoplasma A-116

Test for Neurovirulence in Monkeys A-110

Test for Nucleotide Sequence of SV40 A-77

Test for Reverse Transcriptase Activity A-82

Test for Tablet Friability A-40

Test for Visible Particles A-36

Test Requirements for Calf Serum A-140

Test Requirements for SPF Chicken Embryos A-137

Test Requirements of Microbes for Laboratory
Animals A-137

Test Requirements of Parasites for Laboratory
Animals A-139

Tetanus Antitoxin 153

Tetanus Antitoxin, Freeze-dried 155

Tetanus Vaccine, Adsorbed 71

Typhoid and Paratyphoid A & B Combined Vaccine 34

Typhoid and Paratyphoid A Combined Vaccine 32

Typhoid Vaccine 29

U

Ultraviolet-visible Spectrophotometry A-16

V

Vi Polysaccharide Typhoid Vaccine 36